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Canine and feline uveal melanocytic tumours: Histologic and immunohistochemical characteristics of 32 cases

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Abstract

Objective: Gross, histopathological, and immunohistochemical characteristics of uveal melanocytic neoplasms in dogs and cats were investigated.

Samples: Thirty-two enucleated globes with uveal melanocytic neoplasms, 27 from dogs and 5 from cats, were examined.

Procedures: Morphological characteristics of uveal melanocytic neoplasms in dogs and cats were evaluated with anti-PNL2, anti-Melan-A, anti-Ki-67, anti-caspase-3, and anti-BAP1 immunomarkers. Statistical analysis was performed to compare canine melanocytomas and melanomas.

Results: The 32 uveal neoplasms were classified as melanocytomas (19/27 in dogs) or melanomas (8/27 in dogs, 5/5 in cats). Most tumours (84%) were located in the anterior uvea. Neoplastic cells were classified as epithelioid, spindle-shaped, mixed, or special type (balloon and signet ring cells). The percentage of cells with melanin, melanin concentration within cells, anisocytosis and anisokaryosis, mitotic count, lymphocytic inflammation, necrosis, vascular invasion, and glaucoma were also characterized. Anisocytosis, percentage of neoplastic cells with melanin, mitotic count, and indices (proliferation and apoptotic) varied significantly between canine uveal melanomas and melanocytomas; in general, melanomas had greater cell variability, were less pigmented, and had a higher mitotic count. The melanocytic origin of the neoplasms was confirmed by positive anti-PNL2 immunolabelling (29/32) and positive anti-Melan-A immunolabelling (3/32). In canine uveal melanomas, anisocytosis and anisokaryosis correlated with less pigmentation and minimal pigmentation correlated with a high percentage of immunolabelling for caspase-3.

Conclusions: Uveal melanocytomas were more common in dogs, and uveal melanomas were more frequent in cats. Anisocytosis, percentage of neoplastic cells with melanin, and mitotic count are important histologic characteristics of malignancy to evaluate

in uveal melanocytic neoplasms. The proliferation and apoptotic indices are relevant when comparing malignant tumours with benign tumours.

KEYWORDS
apoptosis, globe, immunohistochemistry, melanocytoma, melanoma, tumour aggressiveness factors

1 | INTRODUCTION

Melanocytic tumours are the most common intraocular neoplasms in dogs (Grahn et al., 2019) and cats (diffuse iris melanoma), (Dubielzig, 2017) with globes being the most frequent site for melanocytic neoplasms in cats (Day & Lucke, 1995; Patnaik & Mooney, 1988). According to the World Health Organization, the term melanocytoma is used for benign neoplasms and melanoma is used for malignant melanocytic neoplasms (Goldschmidt et al., 1998). Uveal melanocytic neoplasms can develop from iridal, ciliary body, and choroidal melanocytes (Hu, 2005). The anterior uvea is the main site for the development of melanocytic neoplasms in animals, with melanocytoma being most frequent in dogs and diffuse iris melanoma the most common in cats (Dubielzig et al., 2010). In cats, prognosis is better when the neoplasm is restricted to the uvea, with no infiltration into adjacent tissues (Kalishman et al., 1998; Miller, 2008; Wiggans et al., 2016).

The diagnosis of uveal melanocytic neoplasia can be challenging due to the highly variable morphology of melanocytes, which can appear similar to cells of other ocular neoplasms, like pigmented iridociliary adenocarcinomas. In melanomas, the morphologic characteristics of neoplastic cells and the quantification of mitoses have prognostic relevance (Dubielzig et al., 2010; Goldschmidt & Goldschmidt, 2017; Spangler & Kass, 2006).

To confirm the diagnosis of melanocytic neoplasms, immunohistochemistry is particularly useful. Positive labelling for Melan-A (Verdijk et al., 2011) and PNL2 confirms the melanocytic lineage of the neoplasm (Smedley et al., 2011; Verdijk et al., 2011; Wiggans et al., 2016). The Melan-A protein, a product of the MART-1 gene, indicates melanocytic differentiation (Chen et al., 1996). Melan-A has cytoplasmic expression and is a good immunomarker to use in the diagnosis of atypical uveal melanomas (Fernandes et al., 2007). Anti-PNL2 is a monoclonal antibody initially developed for the detection of human somatostatin receptors. Expression also occurs in the cytoplasm of melanocytic cells, but the target molecule remains unknown (Aung et al., 2010).

Evaluation of cell proliferation is of prime importance in melanocytic neoplasms because it is one of the most commonly used criteria for malignancy. Ki-67, a well-known proliferation marker, is considered highly sensitive and useful for prognostic evaluation and monitoring of response to treatment (Brown & Gatter, 2002), as demonstrated in non-ocular melanocytic tumours in dogs (Roels et al., 1999; Smedley et al., 2011) and cats (Roels et al., 1999; Sabattini et al., 2018). Studies using Ki-67 immunolabelling of uveal melanocytic neoplasms in dogs and cats, however, have not been published. When the mitotic count is difficult to determine due to excess melanin or extensive apoptosis and necrosis, immunolabelling with anti-Ki-67 can be used to quantify proliferating cells (Bergin et al., 2011; Roels et al., 1999).

Identification and evaluation of apoptotic cells is also important in uveal melanocytic neoplasms. A high apoptotic index, considered to be inversely proportional to the proliferation index, indicates lower growth capacity of the neoplasm (Glinsky et al., 1997); however, there are few studies of apoptosis in canine melanomas (Modiano et al., 1999). Caspase-3, the main enzyme responsible for the cellular deoxyribonucleic acid (DNA) fragmentation and an effector protease in cells undergoing apoptosis, is an important enzyme used to detect apoptotic cells (Glinsky et al., 1997).

Apoptosis and proliferation are regulated by different proteins. Among them, the protein BAP1 stands out in melanomas. The gene responsible for the expression of this protein is located on chromosome 3p21. BAP1 performs the deubiquitination process (Carbone et al., 2013; Jensen et al., 1998) and plays an important role in the regulation of gene expression (Carbone et al., 2013) by inhibiting tumour growth (Jensen et al., 1998). In humans, the grade of malignancy of melanocytic neoplasms is negatively correlated with the degree of BAP1 immunolabelling (Koopmans et al., 2014; Shah et al., 2013; Wiesner et al., 2011). In animals, only one study has been published with anti-BAP1 immunolabelling of melanomas of dogs from different anatomical sites (Jama et al., 2018).

Due to the scarcity of studies of the biological behaviour of uveal melanocytic neoplasms in dogs and cats, this study aimed to evaluate and correlate histologic characteristics of neoplastic melanocytic cells with proliferation and apoptotic indices and association with anti-BAP1 labelling in melanomas and melanocytomas of dogs. This study also describes the clinicopathological and immunohistochemical characteristics of uveal melanomas in cats.

2 | MATERIALS AND METHODS

2.1 | Sampling

Thirty-two enucleated globes with uveal melanocytic neoplasms from dogs and cats from July 2014 through July 2019 were included in this study. Clinical data regarding breed, sex, and age were recorded from all cases.

The study followed the guidelines of the Ethics Committee on the Use of Animals at the Universidade Federal de Minas Gerais (protocol number 262/2014).

2.2 | Histopathology

After enucleation, globes were fixed in 10% neutral buffered formalin for further gross analysis. Subsequently, the globes were routinely processed for histologic evaluation. Sections of 4 μm thickness were stained with haematoxylin and eosin (Luna, 1968) and examined under a standard light microscope.

The anatomic site with neoplastic melanocytes was recorded. Neoplastic cells were further classified as epithelioid, spindle-shaped, or mixed (when both types were present, with a minimum of 10% of either type) (Edge & American Joint Committee on Cancer, 2010; Wilcock et al., 2002). In addition, the presence or absence of neoplastic cells with balloon morphology, signet ring cells, and multinucleated giant cells was also recorded. The percentage of neoplastic cells containing melanin was semi-quantified as 0% (amelanotic neoplasms), 1%–50% (low pigmented neoplasms), and 51%–100% (highly pigmented neoplasms). This semi-quantitative assessment was based on the entire sample and was performed by two experienced pathologists. In addition, neoplasms were graded based on anisocytosis, anisokaryosis, and degree of intracellular melanin concentration (mild; moderate; marked) (semi-quantitative evaluation), following the system adapted from Wiggans et al. (2016). The mitotic count was defined as the number of mitotic figures observed in 10 consecutive high-power fields (HPF) (400×; standard area of 1.96 mm^2) without overlap, starting the count in the field with the highest mitotic count (quantitative evaluation).

Other findings associated with the neoplasms were also recorded, such as the presence or absence of lymphocytic inflammation and intratumoural necrosis (semi-quantitative evaluation), both characteristics were classified as absent, mild, or marked. The development of glaucoma secondary to the neoplasm was also evaluated (Wiggans et al., 2016), as well as any other ocular abnormalities. The diagnosis of glaucoma was based on necrosis and/or loss of ganglion cells and pyknosis of individual cells in the inner nuclear layer of the retina, as well as an increase in the excavation of the optic disc, when visible.

2.3 | Immunohistochemistry

Serial sections of the neoplasms were immunostained with the melanocytic marker PNL2 and, when negative for it, with Melan-A. Additional markers were used for cellular proliferation (Ki-67), cellular apoptosis (caspase-3), and tumour suppression (BAP1). The antibody specifications and the protocols used are shown in Table 1. Tissues were counterstained with Giemsa in order to stain the melanin blue-green (Ramos-Vara, 2005), in an attempt to differentiate the usual brown colour of the melanin from the brown staining of the chromogen diaminobenzidine (DAB). As a negative control, the primary antibodies were replaced with phosphate-buffered saline (Fernandes et al., 2007).

The neoplasms were graded based on the percentage of cells stained with PNL2 (absent to minimal, 0%–9%; mild, 10%–50%; moderate, 51%–75%; and marked, 76%–100%) (Wiggans et al., 2016).

For BAP1, immunolabelling was classified as diffuse (>90%), multifocal to coalescing (10%–90%), or minimal to absent (<10%) (Shah et al., 2013).

For the proliferation index using Ki-67 and the apoptotic index using caspase-3, brown labelling was identified in the nucleus and the cytoplasm, respectively. For both markers, areas of dense staining were selected and a count of immunolabelled cells out of a total of 500 neoplastic cells was performed to determine the proliferation and apoptotic indices in percentages.

2.4 | Statistical analysis

Statistical analyses were performed with the software GraphPad Prism 6.0. The D'Agostino and Pearson omnibus normality test was used to establish whether or not data were normally distributed. *p*-Values of less than 0.05 were considered statistically significant. Possible monotonic relationships of the variables were investigated using a Spearman correlation test. Significant correlations were listed. The sign of the correlation indicates the direction of association between the two variables. χ^2 and Fisher's exact tests were used to compare variables in two-by-two contingency tables to see if they are related. Unpaired *t* tests were used to compare normally distributed data (age, Ki-67 index, and mitotic count). A non-parametric test (Mann–Whitney) was used to compare the non-normally distributed data from caspase immunolabelling. All data are displayed as mean ± SD. The small number of cat samples made statistical comparison unfeasible.

3 | RESULTS

3.1 | Clinical and pathological characteristics

Thirty-two globes with melanocytic neoplasms were collected, 27 from dogs and 5 from cats. Melanocytomas were more common in dogs, whereas the cats of this study had only melanomas ($p = 0.006$).

The clinical and pathological characteristics of canine melanocytomas and melanomas are summarized in Table 2. Of the 27 canine cases, 14 were from male and 13 were from female dogs, 70% were melanocytomas and 30% were melanomas, with no significant difference between the sexes ($p > 0.05$). No amelanotic melanoma was diagnosed in dogs.

Of the 19 dogs with melanocytoma, five of were mixed-breed dogs, two were German shepherd dogs, two had no breed information, and there was 1 each of the following breeds: basset hound, boxer, bulldog, dachshund, Labrador retriever, Lhasa apso, miniature pinscher, Rottweiler, standard schnauzer, and Yorkshire terrier. The group of eight dogs with melanoma was comprised of three Rottweilers, two mixed-breed dogs, one Brazilian terrier, one Poodle, and one had no breed information available. There was no significant difference in the frequency of pure-breed and mixed-breed animals between melanomas and melanocytomas. The age of dogs with melanocytoma ranged from 3 to 14 years, with a mean of 9.7 years, and in dogs with melanoma it

TABLE 1 Primary antibodies and protocols for immunohistochemistry

Antibody (catalogue N°)	Manufacturer	Clonality	Concentration	Antigen retrieval	Incubation (h)	Counterstain	Positive control
PNL2 (sc-59306)	Santa Cruz Biotechnology	PNL2	1:50	Citrate (pH 6.0) + moist heat	16	Giemsa + haematoxylin	Oral melanoma* and uveal melanocytes**
Melan-A (IS633)	DAKO	A103	1:100	Citrate (pH 6.0) + pressurized moist heat	16	Giemsa + haematoxylin	Oral melanoma* and uveal melanocytes**
BAP1 (sc-28383)	Santa Cruz Biotechnology	C-4	1:50	Citrate (pH 6.0) + moist heat	16	Giemsa + haematoxylin	Liver and retinal pigment epithelium**
Ki-67 (M7240)	DAKO	MIB-1	1:50	Citrate (pH 6.0) + pressurized moist heat	16	Giemsa	Oral melanoma***
Caspase-3 (RB-1197-P)	NeoMarkers	CPP32	1:100	Citrate (pH 6.0) + pressurized moist heat	1	Giemsa + haematoxylin	Murine thymus in involution

*Oral melanoma of previously positive dogs (Wiggans et al., 2016).
**Internal control (Koopmans et al., 2014; Wiggans et al., 2016).
***Canine oral melanoma with high cell proliferation index.

ranged from 6 to 14 years, with a mean of 9.3 years, but without a significant difference (Table 2).

Most canine melanocytomas (89%) affected the anterior uvea (iris and ciliary body) and only 11% affected the entire uvea (iris, ciliary body, and choroid). Most melanomas were also located in the anterior uvea (63%) with fewer in the entire uveal tract (37%). The melanomas infiltrated the cornea (7/8), the sclera (6/8), the anterior and posterior chambers and the vitreous space (3/8), blood vessels (2/8), the retina (1/8), the optic nerve (1/8), the retrobulbar musculature (1/8), and also the filtration angle, with obstruction of the aqueous humour flow (4/8) (Table 2) (Figure 1). There was, however, no significant difference in the frequency of melanomas and melanocytomas in the anterior uvea compared with the entire uveal tract.

Canine melanocytoma cell types were classified as mixed, epithelioid, and spindle-shaped, while melanoma cell types were classified as epithelioid and mixed. The frequency of cell patterns was similar between melanomas and melanocytomas.

In canine melanocytomas, nearly all (94%) of the tumours had a high percentage of cells with melanin and most (74%) had marked intracellular melanin concentration. In contrast, the majority (78%) of melanomas had a low percentage of cells with melanin, and only moderate intracellular melanin concentration was observed in 62% of these tumours (Table 2). Canine melanomas had marked anisocytosis compared to the mild anisocytosis of melanocytomas ($p < 0.05$), but there was no significant difference in the degree of anisokaryosis in these tumours (Table 2).

Mild intra-tumoural lymphocytic inflammation was observed in 63% of melanomas and in 58% of melanocytomas. Marked intra-tumoural necrosis was diagnosed in 50% of melanomas and in 5% of melanocytomas; absence of necrosis was diagnosed in 25% of melanomas and 53% of melanocytomas. Glaucoma was present in all dogs with melanoma and in 75% of dogs with melanocytoma. However, there was no significant difference when the association of the degree of inflammation, necrosis, and the presence or absence of glaucoma between melanomas and melanocytomas were assessed. The mean mitotic count was significantly higher in melanomas 19.1 ± 6.0 in 10 HPF compared to melanocytomas 1.2 ± 0.2 in 10 HPF ($p < 0.05$) (Table 2).

The clinicopathological and immunohistochemical characteristics of the uveal melanomas of the 5 cats are summarized in Table 3. The age of the cats ranged from 3 to 11 years, with a mean of 7.8 ± 4.0 years. No melanocytomas or amelanotic melanomas were diagnosed in cats in this study. The melanoma cell types were most often classified as balloon cell, followed by epithelioid, mixed, and signet ring in equal proportions (Figure 2). In feline melanomas, the percentage of melanin pigmented cells and the intracellular melanin concentration ranged from mild to marked. Marked anisocytosis and anisokaryosis were identified in 80% of feline melanomas. In addition, the mean mitotic count of feline melanomas was 9.0 ± 4.0 in 10 HPF; only one had less than seven mitoses in 10 HPF. Intra-tumoural lymphocytic inflammation, when present, was mild and tumour necrosis ranged from mild to marked. In cats, glaucoma was present in 80% of the globes.

3.2 | Comparative immunohistochemical evaluation

The immunohistochemical labelling data of the feline melanomas (Table 3) and canine melanomas and melanocytomas (Table 4) are summarized.

Immunopositivity for PNL2 and Melan-A was identified by the brown labelling in the cytoplasm of neoplastic cells, with at least 10% of labelled cells considered positive (Smedley et al., 2011). Immunopositivity for BAP1 was also identified by the predominantly cytoplasmic and occasionally nuclear brown labelling.

Among the 19 dogs diagnosed with uveal melanocytoma, only one did not have cytoplasmic immunolabelling for PNL2. As for the eight

TABLE 2 Clinical-pathological characteristics of uveal melanocytic neoplasms in 27 dogs

	N°	Melanoma[a]	Melanocytoma[b]
Age[c], mean (SD)			
	24	9.25 (0.97)	9.71 (0.8)
Breeds, N° (%)[c]			
Crossbreed	7	5 (71)	2 (29)
Pure breeds[d]	17	5 (29)	12 (71)
Sex, N° (%)			
Male	14	4 (29)	10 (71)
Female	13	4 (31)	9 (69)
Anatomic location, N° (%)			
Anterior uvea (iris and ciliary body)	22	5 (23)[e]	17 (77)[f]
Entire uveal tract (iris, ciliary body, and choroid)	5	3 (60)	2 (40)
Cellular morphology, N° (%)			
Epithelioid	9	4 (44)	5 (56)
Fusiform	2	0 (0)	2 (100)
Mixed	16	4 (25)	12 (75)
Tumour characteristics, N°(%)			
Anisocytosis[g]			
Mild	17	2 (12)	15 (88)
Moderate	5	1 (20)	4 (80)
Marked	5	5 (100)	0 (0)
Anisocariosis			
Mild	17	1 (6)	16 (94)
Moderate	3	2(67)	1 (33)
Marked	5	5 (100)	0 (0)
% of neoplastic cells with melanin[g]			
1%–50% (low pigmentation)	9	7 (78)	2 (22)
51%–100% (high pigmentation)	18	1 (6)	17 (94)
Melanin concentration			
Mild	3	2 (67)	1 (33)
Moderate	9	5 (56)	4 (44)
Marked	15	1 (7)	14 (93)
Mitotic count, mean (SD)[g]	14	19.13 (5.97)	1.16 (0.16)
Inflammation, N° (%)			
Absent	10	2 (20)	8 (80)
Mild	16	5 (31)	11 (69)
Marked	1	1 (100)	0 (0)
Necrosis, N° (%)			
Absent	12	2 (17)	10 (83)
Mild	10	2 (20)	8 (80)
Marked	5	4 (80)	1 (20)
Glaucoma, N° (%)			
Absent	3	0 (0)	3 (100)
Present	14	5 (36)	9 (64)

Abbreviation: N°, number.

[a]Some data were not available or an evaluation was not possible for all eight canine melanomas.

[b]Some data were not available or an evaluation was not possible for all 19 canine melanocytomas.

[c]Three dogs with unknown breed.

[d]Pure breeds included German shepherd, basset hound, boxer, bulldog, dachshund, Labrador retriever, Lhasa apso, miniature pinscher, Rottweiler, standard schnauzer, and Yorkshire terrier.

[e]In one dog, the neoplasm infiltrated the peripheral portion of the choroid.

[f]In 10 dogs, the neoplasm expanded to the peripheral portion of the choroid.

[g]Significant difference ($p < 0.05$) in exact of fisher or χ^2 test or unpaired t tests.

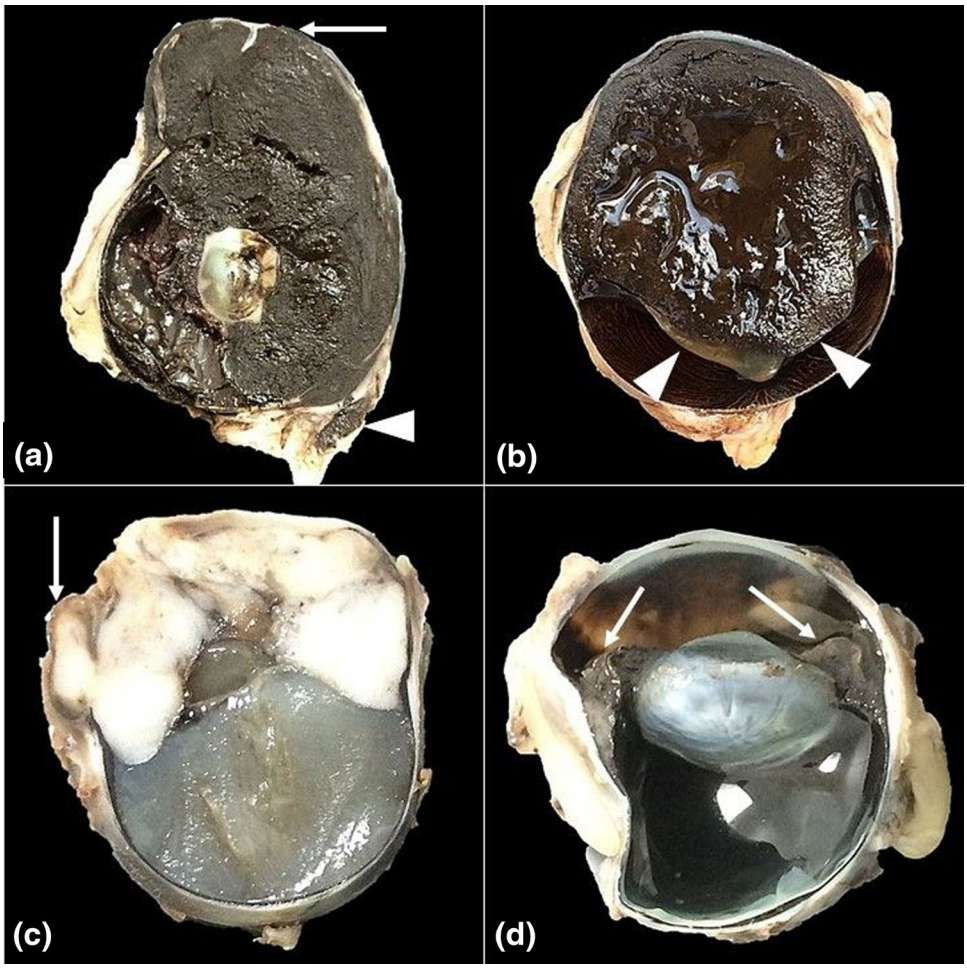

FIGURE 1 Gross images of uveal melanocytic neoplasia in the globes of dogs and cats. (a) Panuveal melanocytoma in an 8-year-old male mixed-breed dog. The markedly pigmented neoplasm fills the anterior and posterior compartments, extensively infiltrates the corneal (arrow) and extraocular region (arrowhead), and causes iridocorneal angle obstruction and posterior lens luxation. (b) Anterior uveal melanoma in a 12-year-old female miniature pinscher dog. This markedly pigmented neoplasm, which effaces the anterior compartment, has peripheral choroidal invasion, iridocorneal angle obstruction and retinal detachment (arrowheads). (c) Anterior uveal melanoma in an 8-year-old male mixed-breed dog. This poorly pigmented neoplasm effaces the anterior compartment, with corneal and sclera invasion (arrow) and iridocorneal angle obstruction. (d) Diffuse iris melanoma in a 10-year-old male Persian cat. This neoplasm is moderately pigmented, causing diffuse thickening of the iris and ciliary body (arrows)

dogs with melanomas, six were PNL2 positive and two negatives. The three melanocytic neoplasms in dogs that were negative for PNL2 had positive cytoplasmic labelling with Melan-A. All feline melanomas were positive for PNL2.

The frequency (percentage of cells) and degree (intensity) of PNL2 labelling in canine melanomas and melanocytomas were similar. Marked PNL2 labelling was observed in 63% of melanocytomas and in 25% of melanomas. In cats, the labelling was marked in 80% of melanomas (Figure 3).

All 32 (100%), canine and feline melanocytic neoplasms were positive for BAP1, and most of the labelling was in the cytoplasm. There was no difference in the degree of labelling for BAP1 between canine melanomas and melanocytomas. Multifocal to coalescing labelling for BAP1 was observed in 74% of melanocytomas compared to an even split between multifocal to coalescing and diffuse immunolabelling in the melanomas. Feline melanomas had 60% multifocal to coalescing and 40% diffuse immunolabelling (Figure 4).

The proliferation index in canine neoplasms, determined by nuclear immunolabelling of neoplastic cells with Ki-67 antibody, ranged from 3% to 12% in melanocytomas, with a mean of 6.5 ± 2.1. In melanomas nuclear immunolabelling ranged from 16% to 70 % with a mean of 38.3 ± 12.6 ($p < 0.05$) (Table 4). The cell proliferation index in feline melanomas ranged from 11% to 30%, with a mean of 19.0 ± 7.4 (Figure 5).

Cytoplasmic immunolabelling for apoptosis in dogs with anti-caspase-3 antibody ranged from 4% to 50% in melanocytomas, with a median of 14% and, in melanomas the range was 30%–84% with a median of 58% ($p < 0.05$) (Table 4). In cats, the expression of caspase-3 in melanomas ranged from 4% to 92%, with a mean of 23.2 ± 38.6 (Figure 6).

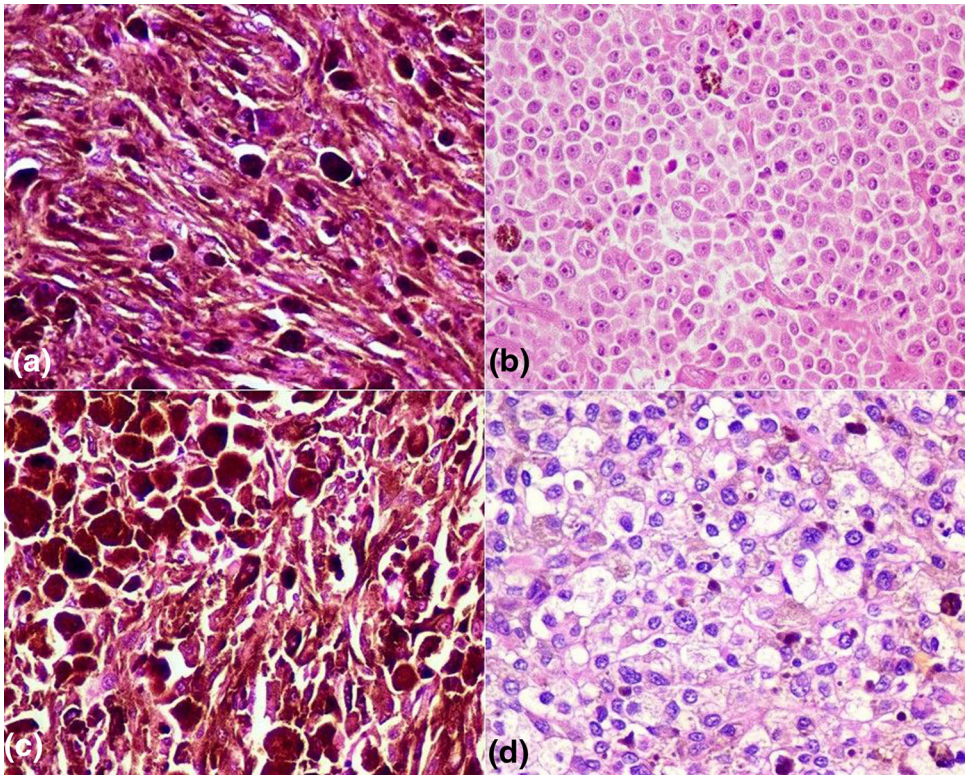

FIGURE 2 Histologic characteristics of uveal melanocytic neoplasms in dogs and cats. (a) Spindle cell melanocytoma in a 12-year-old female miniature pinscher dog; (b) epithelioid cell melanoma in an 8-year-old male mixed-breed dog; (c) mixed cell melanocytoma in an 8-year-old male mixed-breed dog; (d) balloon cell melanoma in an 11-year-old female Persian cat. (Hematoxylin and eosin, 400×)

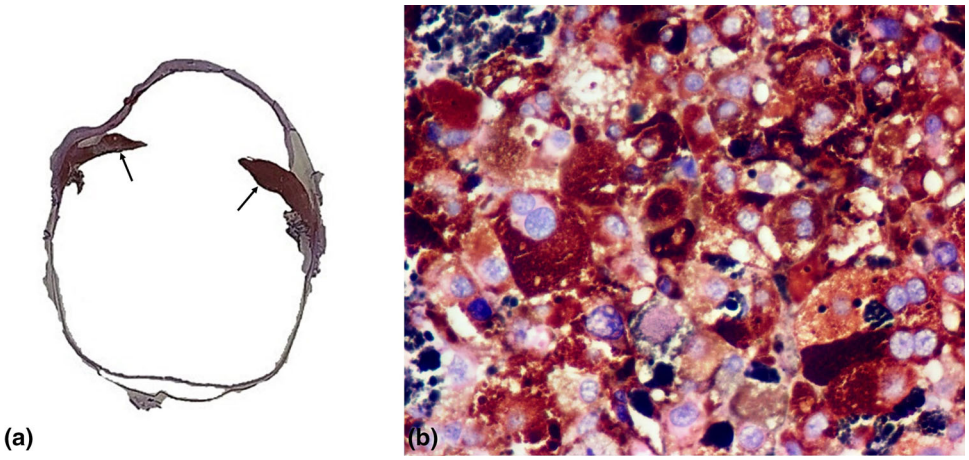

FIGURE 3 Anti-PNL2 immunolabelling of uveal melanocytic neoplasms in cats. (a) Diffuse iris melanoma in a 4-year-old female mixed-breed cat, with diffuse and strong labelling in the iris and ciliary body (arrows) (subgross). (b) Higher magnification of the neoplasm in image a demonstrating the cytoplasmic labelling (400×). For all images, the diaminobenzidine (DAB) chromogen (brown) was used, contrasting with the melanin (blue-green). Counterstaining: Giemsa and haematoxylin

The proliferation index could not be determined in 19 dogs (four melanomas and 15 melanocytomas), and the apoptotic index could not be determined in 12 dogs (three melanomas and 9 melanocytomas). Despite the use of Giemsa as the counterstain to avoid that the melanin masks the chromogenic IHC labelling, the abundance of intracellular melanin concentration nonetheless prevented accurate immunohistochemical analysis of these cases.

TABLE 3 Clinical-pathological and immunohistochemical characteristics of uveal melanomas in five cats

Signalment	Histological characteristics								Immunohistochemical characteristics	
Breed/sex/age	Anatomical location	Cell morphology	Cells with melanin	Intracellular melanin concentration	Anisocytosis	Anisokaryosis	Lymphocytic inflammation	Necrosis	Glaucoma	PNL2	BAP1
Mixed-breed/male/11 years	Anterior uvea (iris and ciliary body)	Epithelioid	51%–75%	Marked	Marked	Marked	Absent	Absent	Present	Marked (76%–100%)	Multifocal to coalescing (10%–90%)
Persian/male/10 years	Anterior uvea (iris and ciliary body)	Mixed	26%–50%	Moderate	Marked	Marked	Mild - multifocal	Mild - multifocal	Present	Marked (76%–100%)	Diffuse (> 90%)
Persian/female/11 years	Anterior uvea (iris and ciliary body)	Balloon cell[a]	1%–25%	Mild	Moderate	Moderate	Absent	Absent	Present	Marked (76%–100%)	Diffuse (> 90%)
Mixed-breed/female/4 years	Anterior uvea (iris and ciliary body)	Balloon cell[a]	26%–50%	Moderate	Marked	Marked	Mild - multifocal	Absent	Absent	Marked (76%–100%)	Multifocal to coalescing (10%–90%)
Angora/male/3 years	Anterior uvea (iris and ciliary body)	Signet ring cell	26%–50%	Mild	Marked	Marked	Mild - multifocal	Marked - multifocal to coalescent	Present	Mild (10%–50%)	Multifocal to coalescing (10%–90%)

[a]Cats with more than one morphological type (giant multinucleated cells and signet ring cells).

TABLE 4 Immunohistochemical characteristics of uveal melanocytic neoplasms in 27 dogs

	No.	Melanoma[a]	Melanocytoma[b]
PNL-2, No (%)			
Absent to minimal (0%–9%)	3	2 (67)	1 (33)
Mild (10%–50%)	5	3 (60)	2 (40)
Moderate (51%–75%)	5	1 (20)	4 (80)
Marked (75%–100%)	14	2 (14)	12 (86)
BAP-1, No (%)			
Multifocal to coalescing (10%–90%)	18	4 (22)	14 (78)
Diffuse (90%–100%)	9	4 (44)	5 (56)
Ki-67, mean, (SD)[c]	8	38.3 (12.61)	6.45 (2.05)
Caspase (median)[d]	15	58.4	13.9

[a]Some data were not available or an evaluation was not possible for all eight canine melanomas.
[b]Some data were not available or an evaluation was not possible for all 19 canine melanocytomas.
[c]significant difference ($p < 0.05$) in unpaired *t* test.
[d]significant difference ($p < 0.05$) in Mann–Whitney test.

3.3 | Correlation between morphologic and immunohistochemical parameters

In canine melanomas, a strong positive correlation was found between anisokaryosis and anisocytosis ($r_s = 0.97$ and $p = 0.02$). Anisocytosis was negatively associated with the percentage of cells with melanin ($r = -0.28$, $p < 0.001$) and intracellular melanin concentration ($r_s = -0.47$, $p < 0.001$). A similar trend was observed between anisokaryosis and percentage of cells with melanin ($r = -0.38$, $p < 0.001$), and intracellular melanin concentration ($r_s = -0.52$, $p < 0.001$). A low-to-moderate negative correlation was found between the percentage of cells with melanin and expression pattern of BAP-1 ($r_s = -0.37$, $p < 0.001$), and percentage of caspase-3 labelling ($r = -0.70$, $p < 0.001$) and cell morphology ($r_s = -0.37$, $p < 0.001$) in canine melanomas. In addition, a strong negative correlation was found between the intracellular melanin concentration and percentage of caspase-3 labelling ($r_s = -0.89$, $p < 0.001$).

In canine melanocytomas, a moderate positive correlation was found between anisokaryosis and anisocytosis ($r_s = 0.45$, $p < 0.001$). A similar trend was observed between the intracellular melanin concentration and percentage of cells with melanin ($r = 0.63$, $p < 0.001$) and between anisokaryosis and necrosis ($r_s = 0.46$, $p < 0.001$). In addition, a moderate to strong negative correlation was found between percentage of cells with melanin and expression pattern BAP-1 ($r_s = -0.57$, $P < 0.001$), cell proliferation index (Ki-67) ($r_s = -0.77$, $p < 0.001$), and percentage of caspase-3 labelling ($r_s = -0.52$, $p < 0.001$). A similar trend was observed between the intracellular melanin concentration and cell proliferation index (Ki-67) ($r_s = -0.77$, $p < 0.001$) and percentage of caspase-3 labelling ($r_s = -0.74$, $p < 0.001$). The mitotic

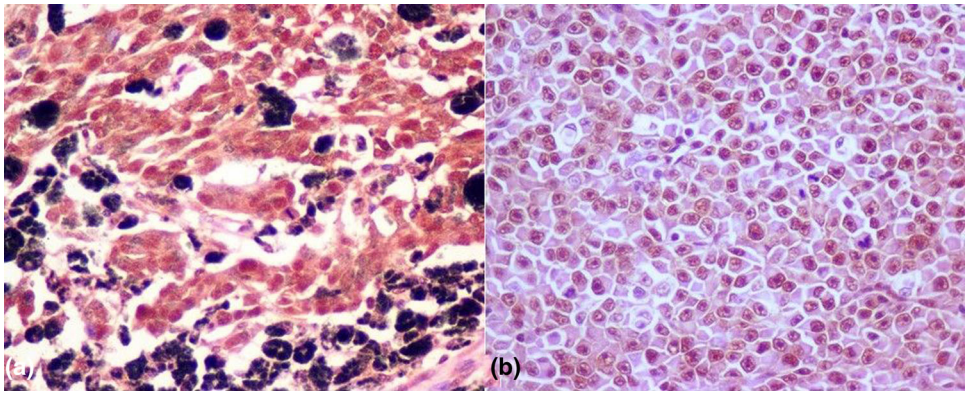

FIGURE 4 Anti-BAP1 immunolabelling of uveal melanocytic neoplasms in dogs and cats. (a) Melanoma in a 12-year-old male Rottweiler dog, with multifocal to coalescing nuclear and cytoplasmic labelling. (b) Melanoma with low pigmentation in a 3-year-old male Angora cat, with multifocal to coalescing nuclear labelling. For all images, the diaminobenzidine (DAB) chromogen (brown) was used, contrasting with the melanin (blue-green). Counterstaining: Giemsa and haematoxylin (400×)

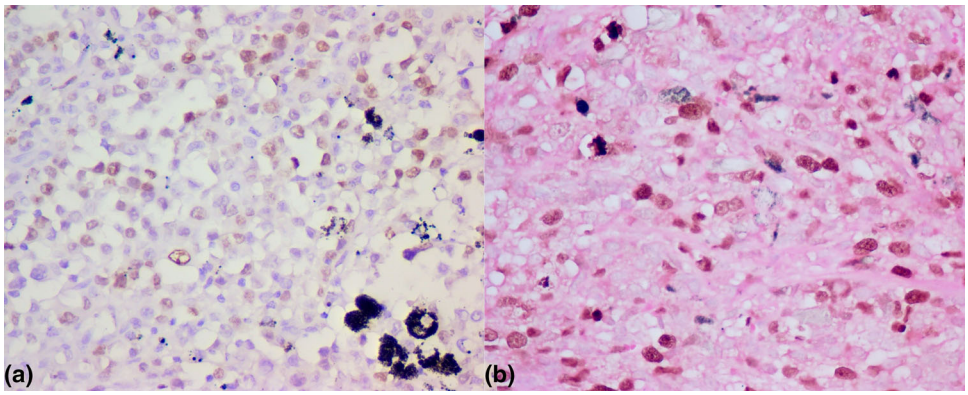

FIGURE 5 Anti-Ki-67 immunolabelling of uveal melanocytic neoplasms in dogs and cats. (a) Melanoma in a 6-year-old male Rottweiler dog, with a high proliferation index; (b) melanoma in an 11-year-old female Persian cat, with high proliferation index. (a) Counterstaining: haematoxylin; (b) counterstaining: Giemsa. For all images, the diaminobenzidine (DAB) chromogen (brown) was used, and the labelling is nuclear

count was also negatively correlated with inflammation ($r = -0.63$, $p < 0.001$), necrosis ($r_s = -0.44$, $p < 0.001$), expression pattern of BAP-1 ($r_s = -0.31$, $p < 0.001$), and percentage of caspase-3 labelling ($r_s = -0.70$, $p < 0.001$). A similar trend was found between the percentage of caspase-3 labelling and cell morphology ($r_s = -0.59$, $P < 0.001$) and anisocytosis ($r_s = -0.52$, $p < 0.001$).

4 | DISCUSSION

This is the first study that compares benign and malignant uveal melanocytic neoplasms in dogs and cats with extensive histologic and immunohistochemical characteristics, including aspects of cell proliferation (Ki-67) and apoptosis (caspase-3). The association of a high proliferation index and a low apoptotic index in uveal melanomas is indicative of their aggressiveness and malignant behaviour.

The morphological characteristics of neoplastic melanocytes associated with anti-PNL2 and anti-Melan-A markers are important in the diagnosis of uveal melanocytic neoplasms and corroborate some previous studies (Smedley et al., 2011; Wiggans et al., 2016). The detection of BAP1 does not appear to have any prognostic relevance in dogs and cats, similar to previous findings in cutaneous, digital, periocular, and uveal melanomas in dogs (Jama et al., 2018).

In this study, the morphological aspects of the neoplastic cells were consistent with the diagnosis of melanocytic neoplasms, but immunolabelling with anti-Melan-A (Verdijk et al., 2011) and anti-PNL2 antibodies allowed further confirmation of the melanocytic origin of neoplasms (Smedley et al., 2011; Verdijk et al., 2011; Wiggans et al., 2016). Interestingly, two globes had no cytoplasmic immunolabelling with anti-PNL2, requiring the use of anti-Melan-A for confirmation. These results demonstrate that, when uveal melanomas are negative for PNL2 and melanocytic origin is strongly suspected, this diagnosis should not be dismissed and the sample should be submitted to Melan-A immunolabelling (Giudice et al., 2010).

In the present study, uveal melanocytomas in dogs occurred more frequently than melanomas, which is consistent with previous reports (Dubielzig et al., 2010; Wilcock & Peiffer, 1986). Approximately 30% of the canine melanomas in our study occurred in the anterior uvea, which

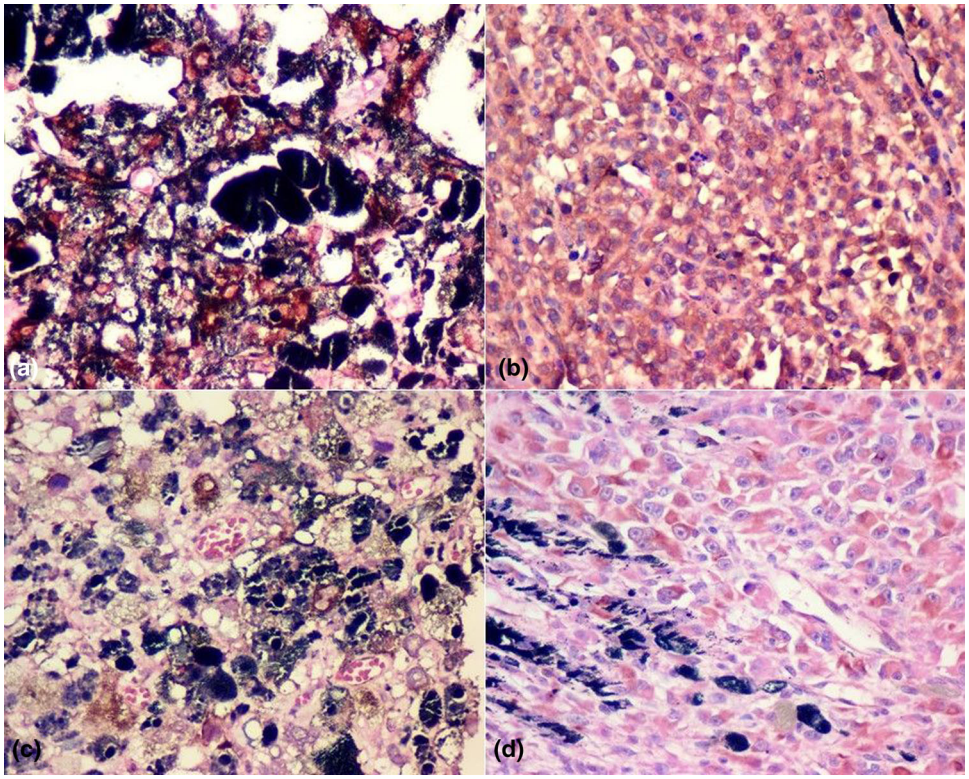

FIGURE 6 Anti-caspase-3 immunolabelling of uveal melanocytic neoplasms in dogs and cats. (a) Melanocytoma in a 13-year-old female basset hound dog, with a low apoptotic index; (b) melanoma in a 6-year-old, male Rottweiler dog, with a high apoptotic index; (c) melanoma in a 4-year-old female mixed-breed cat, with a low apoptotic index; (d) melanoma in a 3-year-old male Angora cat, with a high apoptotic index. For all images, the diaminobenzidine (DAB) chromogen (brown) was used, contrasting with the melanin (blue-green). Counterstaining: Giemsa and haematoxylin (400×)

is twice that reported in other studies (approximately 15%) (Wilcock & Peiffer, 1986). In contrast, all uveal melanocytic neoplasms in cats were malignant, which is in agreement with previous studies (Dubielzig et al., 2010).

Melanocytic neoplasms in dogs in this study were most often found in the anterior uvea, similar to other reports (Dubielzig et al., 2010). However, in several of the dogs with melanocytoma, neoplastic cells had expanded to the peripheral portion of the choroid. In two dogs, the distribution was diffuse in the uvea. Therefore, melanocytomas despite benign can still have severe intraocular consequences, for example, lens luxation and secondary glaucoma (Wilcock & Peiffer, 1986). In canine melanomas, there was invasion of neoplastic cells into the cornea, sclera, retina, blood vessels, retrobulbar muscles, and the optic nerve, which has previously indicated a poor prognosis (Miller, 2008); in contrast to another study, in which tumour infiltration was not considered a reliable predictor of survival (Giuliano et al., 1999). In cats, melanomas occur mainly in the irideal stroma, with infiltration of the ciliary body which has been reported as a risk factor for metastasis due to the presence of vessels in this tissue (Kalishman et al., 1998). Some cats in this study had infiltration of neoplastic cells into the cornea, sclera, and retina. Choroid invasion, which predisposes to the occurrence of metastasis (Wiggans et al., 2016), was not found in this study.

In many dogs and cats of this study, neoplastic cells obstructed the iridocorneal angle, resulting in the non-drainage of aqueous humour and secondary glaucoma (Kalishman et al., 1998). After glaucoma is established, the ocular change can become evident to the owner if the globe is enlarged. This can result in presentation to a veterinarian for evaluation; however, this often occurs at an advanced stage of disease and results in enucleation, as seen in the dogs and cats of this study. If enucleation is late, metastases may have already occurred (Delgado et al., 2016; Hyman et al., 2005).

Melanocytomas have more pigmentation, with epithelioid and spindle-shaped cells and less than four mitoses in 10 HPF. While melanomas are less pigmented or amelanotic, with marked cellular and nuclear pleomorphism and more than four mitoses in 10 HPF (Dubielzig et al., 2010). Reported findings are similar to our findings for uveal melanomas in this study. Cells with epithelioid and mixed characteristics were only found in canine melanomas. In cats, balloon cells were the most frequent.

In the canine and feline uveal melanomas in our study, there was also inflammation and necrosis, which have been proposed as indicators of metastasis and poor prognosis (Spangler & Kass, 2006; Wiggans et al., 2016). In those studies, intra-tumoural necrosis was associated with a more aggressive phenotype and considered a risk factor for metastasis.

Some authors recommend that canine uveal melanocytic neoplasms should be considered malignant when they exceed four mitoses in 10 HPF (Dubielzig et al., 2010). The canine melanocytomas in the current study were therefore diagnosed as such based on less than or equal

to three mitoses and the melanomas based on equal to or more than four mitoses in 10 HPF. Cats with diffuse iris melanoma with less than seven mitoses in 10 HPF were previously associated with a reduced metastatic rate (Wiggans et al., 2016). Of the five cats in our study, only one had less than seven mitoses in 10 HPF.

The immunolabelling for Ki-67 allowed a more sensitive determination of the proliferation index of cells (Bergin et al., 2011; Roels et al., 1999). In the present study, canine (70%) and feline (30%) uveal melanomas had high cell proliferation rates, indicating high tumour aggressiveness (Weinstein et al., 2014) and metastatic potential (Brown & Gatter, 2002). The mean proliferation index of canine melanocytomas was 6.4%. These results agree with other studies, which determined that dogs with extraocular melanomas have a poor prognosis when the proliferation index is greater than or equal to 15% (Smedley et al., 2011). Apoptosis studies in melanocytic neoplasms are scarce, with no reports on the use of apoptotic markers in uveal neoplasms in animals. In this study, a high apoptotic index was found in both melanocytomas and melanomas, contradicting the human literature for these tumours, which reports decreased apoptotic indices in melanomas (Glinsky et al., 1997).

Tumour suppression was also tested in this study with BAP1 protein immunolabelling. The use of BAP1 in uveal melanocytic neoplasms in dogs and cats was not useful because there was no difference in the intensity of the labelling among benign and malignant neoplasms. These results are similar to one study (Jama et al., 2018) and in contrast with other three studies (Koopmans et al., 2014; Shah et al., 2013; Wiesner et al., 2011), which described decreased labelling in human malignant neoplasms. It is noteworthy that, in some intensely pigmented neoplasms, low immunolabelling for BAP1 was obtained. The location of the BAP1 labelling in the present study was cytoplasmic, in contrast to observations in uveal melanomas in humans (Kalirai et al., 2014) and as reported in uveal and oral melanomas in dogs, of which all had positive nuclear and negative cytoplasmic labelling (Jama et al., 2018). In those reports, the labelling was both cytoplasmic and nuclear. In other studies of melanomas in humans, there was exclusively nuclear labelling (Shah et al., 2013, Koopmans et al., 2014). Based on these differences, the human BAP1 protein antibody may not be valid for dogs and cats. More studies are recommended to evaluate the use of this antibody in dogs and cats, following the validation methods described by Caswell et al. (2018).

The lack of the follow-up information about the animals in this study is a limitation. Attempts to contact the owners and veterinarians responsible for each animal did not yield additional information. Therefore, more studies are needed to assess the occurrence of metastases post-enucleation, survival, and prognosis of animals with uveal melanocytic neoplasms.

The findings of the current study revealed a higher frequency of melanocytoma in dogs and a trend in the occurrence of melanoma in cats. The degree of anisocytosis, percentage of neoplastic cells with melanin, mitotic count, proliferation index (Ki-67), and apoptotic index (caspase-3) is important histologic and immunohistochemistry characteristics to evaluate for establishing malignant potential in uveal melanocytic neoplasms in dogs. This study also suggests that the detection of BAP1 protein in melanocytic neoplasms in dogs and cats is not correlated with the malignant potential of the neoplasm or with the proliferation and apoptotic indices. Thus, our findings on the clinic morphological and immunohistochemical characteristics of uveal melanocytic neoplasms are important for the evaluation of malignancy of these neoplasms and will be useful for future studies on survival and prognosis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization, data curation, formal analysis, investigation, methodology, resources, visualization, writing—review, and editing: Matheus Vilardo Loés Moreira, Roselene Ecco, Ingeborg Maria Langohr, Marina Rios de Araújo Campos. *Conceptualization, investigation, methodology, visualization, writing—review, and editing*: Matheus Vilardo Loés Moreira, Roselene Ecco, Ingeborg Maria Langohr, Enio Ferreira. *Investigation, visualization, writing—review, and editing*: Matheus Vilardo Loés Moreira, Roselene Ecco, Ingeborg Maria Langohr, Guilherme Reis Blume. *Conceptualization, data curation, investigation, resources, writing—review, and editing*: Fabiano Montiani-Ferreira. *Conceptualization, data curation, formal analysis, funding acquisition, methodology, project administration, resources, supervision, validation, visualization, writing—review, and editing*: Roselene Ecco, Ingeborg Maria Langohr.

ETHICS STATEMENT

The study followed the guidelines of the Ethics Committee on the Use of Animals at the Universidade Federal de Minas Gerais (protocol number 262/2014).

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