UNIVERSITI PUTRA MALAYSIA

COMPARATIVE HISTOPATHOLOGICAL ANALYSIS OF SOLAR-INDUCED CHANGES IN CANINE CUTANEOUS HAEMANGIOMA AND HAEMANGIOSARCOMA

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It is hereby certified that we have read this project paper entitled "**COMPARATIVE HISTOPATHOLOGICAL ANALYSIS OF SOLAR-INDUCED CHANGES IN CANINE CUTANEOUS HAEMANGIOMA AND HAEMANGIOSARCOMA**" by Aneetta Puvaneswaran Chettiar and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD4999 - Final Year Project.

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Dedicated to my family, friends, Max, and Ginger.

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HSA	haemangiosarcoma
HA	haemangioma
cHSA	cutaneous haemangiosarcoma
cHA	cutanenous haemangioma
UV	ultraviolet
IHC	immunohistochemistry
VEGF	vascular endothelial growth factor
PTEN	phosphatase and tensin homolog
ras	rat sarcoma
GSD	German shepherd dog
MST	median survival time
UVA	ultraviolet A
UVB	ultraviolet B
ROS	reactive oxygen species
DNA	deoxyribonucleic acid
bFGF	basic fibroblast growth factor
ST	survival time
HE	haematoxylin and eosin
UPM	Universiti Putra Malaysia
FFPE	formalin-fixed paraffin-embedded
CPD	cyclobutene pyrimidine dimers
COX-2	cyclooxygenase-2
ECM	extracellular matrix
MC	mitotic count

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD-4999-Projek

ANALISIS PERBANDINGAN HISTOPATOLOGI PERUBAHAN AKIBAT SINARAN MATAHARI PADA HAEMANGIOMA DAN HAEMANGIOSARKOMA KULIT ANJING

oleh

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2023

Penyelia: Prof. Madya Dr. Gayathri Thevi Selvarajah

Haemangioma kulit (cHA) dan haemangiosarkoma kulit (cHSA) biasanya berkembang dalam jenis anjing yang mempunyai bulu pendek dan kulit yang kurang berpigmen disebabkan oleh pendedahan berterusan kepada sinaran matahari. Kesan pendedahan kepada matahari boleh dilihat melalui perubahan histopatologi akibat sinaran matahari, iaitu sel-sel sunburn, elastosis solar, keratosis aktinik, dan fibrosis dermal. Tujuan kajian ini adalah untuk membandingkan perubahan histopatologi akibat sinaran matahari antara cHA dan cHSA pada anjing, menjelaskan kesan pendedahan matahari yang berpanjangan terhadap perkembangan tumor dan hubungannya dengan ciri keganasan dalam cHSA. Blok tisu cHA (n = 10) dan cHSA (n = 20) yang disahkan di Makmal Histopatologi, Fakulti Perubatan Veterinar, UPM dari tahun 2018 hingga 2023 dinilai untuk perubahan histopatologi akibat sinaran matahari, keradangan, kedalaman invasi tumor, dan dinilai mengikut grednya.

Hubungan antara perubahan akibat sinaran matahari dengan ciri-ciri keganasan seperti pengiraan mitosis, pembezaan tumor, dan kedalaman invasi dinilai dalam kumpulan cHSA. Di antara empat perubahan akibat sinaran matahari, fibrosis dermal didapati mempunyai pengesanan yang signifikan ($P<0.05$) dalam cHSA. Kedalaman invasi tumor menunjukkan kepentingan antara jenis tumor, dengan lebih banyak invasi ke dalam hipodermis diperhatikan dalam cHSA. Infiltrat radang didapati secara signifikan lebih tinggi dalam cHSA ($P<0.05$). Tiada hubungan ditemui antara perubahan akibat sinaran matahari dan ciri-ciri keganasan dalam kohort tumor cHSA ini. Neoplasma vaskular kulit adalah spektrum berterusan dari yang benign kepada yang malignan. Kajian ini memberikan pencerahan baru berkenaan ciri-ciri histologi cHSA yang berkaitan dengan sinaran UV pada anjing. Khususnya, fibrosis dermal merupakan salah satu perubahan akibat sinaran matahari, dan perubahan histopatologi lain termasuk infiltrat sel- sel radang, pengiraan mitosis, dan kedalaman invasi sel neoplasia ke dalam hipodermis adalah antara ciri-ciri berguna untuk membezakan cHSA dari cHA, dan mungkin boleh membantu dalam mengenalpasti transformasi malignan awal neoplasma vaskular kulit.

Kata kunci: haemangioma, kulit; haemangiosarcoma, kulit; anjing; histopatologi; sinaran matahari; malignan

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999 - Project.

COMPARATIVE HISTOPATHOLOGICAL ANALYSIS OF SOLAR-INDUCED CHANGES IN CANINE CUTANEOUS HAEMANGIOMA AND HAEMANGIOSARCOMA

by

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2023

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Cutaneous haemangioma (cHA) and haemangiosarcoma (cHSA) develop mainly in dog breeds with short-haired coats and lightly pigmented skin due to chronic exposure to solar radiation. The effect of solar exposure can be seen via solar-induced histopathological changes, namely sunburn cells, solar elastosis, actinic keratosis, and dermal fibrosis. The aim of this study was to compare solar-induced histopathological changes between canine cHA and cHSA, elucidating the effect of chronic solar exposure on tumour progression and its association with malignancy features in cHSA. Tissue blocks of canine cHA (n = 10) and cHSA (n = 20) diagnosed at the Histopathology Laboratory of the Faculty of Veterinary Medicine, UPM from 2018 to 2023 were evaluated for solar-induced histopathological changes, inflammation, tumour invasiveness and graded accordingly. The associations between solar-induced changes with malignancy features such as mitotic count, tumour differentiation and depth of invasion were assessed within cHSA group.

Among the four solar-induced changes, dermal fibrosis was found to have significantly higher detection (P<0.05) in cHSA. Tumour invasiveness displayed significance between the tumour types, with a higher proportion of invasion into the hypodermis observed in cHSA. Inflammatory infiltrates were detected significantly higher in cHSA (P<0.05). There was no association found between solar-induced changes and malignancy features in this cohort of cHSA tumours. Cutaneous vascular neoplasms are a continuous spectrum ranging from benign to highly malignant. This study provides new insight into the histological characteristics of UV-associated cHSA in dogs. Notably, dermal fibrosis as one of the solar-induced changes, and other histopathology changes including inflammatory infiltrate, mitotic count, and depth of invasion of neoplastic cells into the hypodermis were useful features for differentiating cHSA from HA, and may help in detecting early malignant transformation of cutaneous vascular neoplasms.

Keywords: cutaneous, haemangioma; cutaneous, haemangiosarcoma; canine; solar radiation; histopathology; malignant

1.0 INTRODUCTION

Haemangiosarcoma (HSA) is a common canine malignant neoplasm originating from vascular endothelial cells, while haemangioma (HA) is the benign form of the tumour. HSA can mainly be divided into the visceral and non-visceral forms, where the non-visceral form includes four primary subtypes according to biological differences: actinic cutaneous, non-actinic cutaneous, subcutaneous, and intramuscular HSA (De Nardi et al., 2023). Canine HSA usually develops with highest incidence in the spleen, right atrium, skin, and liver, and commonly metastasizes to the lung and liver (Fernandes and De Nardi, 2008; Oksanen 1978; Brown et al., 1985). Grossly, HSAs are usually non-encapsulated, poorly circumscribed, and friable (Withrow et al., 2013). Compared to visceral HSA, cutaneous and subcutaneous HSA tend to be less aggressive and have a lower propensity to metastasize.

Certain breeds have been reported to be predisposed to the development of cutaneous HSAs (cHSA), such as the Whippet, Italian Greyhound, Beagle, Dalmatian, American Staffordshire Terrier/Pitbull, Boxer, and Basset hound (Hargis et al., 1992; Ward et al., 1994; Szivek et al., 2012). This is suggested to be due to solar radiation as a predisposing factor in dogs with short and sparse hair coats and lightly pigmented skin (Hargis et al., 1992; Nikula et al., 1992). In a study, ultraviolet (UV) radiation exposure has proven to induce the development of cHSA in 48 beagles, where 42 of them were presented with histopathologic change of solar dermatosis, as a solar-induced change (Nikula et al., 1992). Grossly, cutaneous, or subcutaneous HSAs are usually presented

as solitary or multifocal tumours, most often developing on the ventral aspect of the body in dogs and cats (Ward et al., 1994; Szivek et al., 2012; McAbee et al., 2005). One study has suggested that intramuscular and subcutaneous HSAs carried poor prognosis with low survival time, while another study reported a significant difference in survival time in dogs with HSA limited to the dermis (780 days), compared to those with hypodermis (172 days) and underlying muscle involvement (307 days) (Ward et al., 1994; Shiu et al., 2011). Dogs with the actinic subtype of cHSA has also been reported to have increased survival time as tumour growths tend to be less aggressive with lower metastasis rate as compared to the non-actinic subtype which is more aggressive with higher metastasis rate and reduced survival time (Nobrega et al., 2019; Szivek et al., 2012).

To obtain a definitive diagnosis of the visceral forms of HSA and HA, histopathological examinations of tissues are required. In cases where histopathological features are insufficient for accurate diagnosis, immunohistochemistry (IHC) can be performed with markers such as von Willebrand's factor, CD31/platelet endothelial cell-adhesion molecule and vascular endothelial growth factor (VEGF) to assist in diagnosis (Gamlen et al., 2008; Miller et al., 1992; Von Beust et al., 1988; Ferrer et al., 1995; Jakab et al., 2009; Sabattini and Bettini, 2009; Giuffrida et al., 2017; Campos et al., 2012). Routine diagnostics generally consists of laboratory tests such as complete blood count and serum biochemistry panel, cytologic evaluation of any effusions, radiography, and ultrasound (Griffin et al., 2021). Common complete blood count findings include anaemia and thrombocytopenia due to haemorrhage (Childress, 2012; Hargis and Feldman, 1991; Hammer et al., 1991; Hirsch et al., 1981). Abdominal ultrasounds are

indicated for dogs with suspected visceral HSA and possible metastatic disease, while thoracic radiography can be carried out to screen for pulmonary metastatic disease (Withrow et al., 2013; Griffin et al., 2021).

As for the cutaneous form of the tumour, though histopathology is also routinely used for diagnosis, it remains a challenge to diagnose and differentiate HA from HSA in histopathologic examination as the tumour progression is a spectrum, and cutaneous HA may develop into HSA. Besides, in cases where tumour growths are presented in a disseminated manner, it can be tough to tell if the HSAs had primarily developed on the skin or as metastases from tumours in visceral organs (Hargis et al., 1992). Actinic change occurs progressively and dogs that initially develop HA may undergo malignant transformation into HSA (Hargis et al., 1992). One study also reported HSAs developing from preexisting HA in skin that was exposed to the sun, further solidifying the suggestion that benign dermal tumours may progress to malignant vascular tumours (Hargis et al., 1992). The association of UV radiation with the development of cutaneous tumours such as squamous cell carcinoma, and to a lesser extent in cutaneous HSAs has been studied, and histopathological findings such as solar elastosis, dermal fibrosis, dermatitis, and epidermal dysplasia were considered actinic changes in cHSA (Griffin et al., 2021; Szivek et al., 2012). A study reported that the majority of cHSA cases had actinic change as detectable UV damage change, confirming the role of solar exposure on tumour development (Szivek et al., 2011). Since the actinic subtype of cHSA has been reported to have lower aggressiveness and better prognosis, thus it is expected that those tumours have a lower grade as histological grading systems encompasses parameters that correlate with neoplasm

aggressiveness (Avallone et al., 2021).

There are currently limited studies examining the histopathological changes in cutaneous HA and HSA caused by solar exposure. To date, there are also no studies investigating the relationship between solar exposure and the development of cutaneous HA or HSA in dogs in Malaysia, where the climate is tropical, with UV radiation index (UVI) as high as 10 to 13 reported throughout the year (World Health Organization). Thus, this aspect of the study is necessary to explore the specific solar-induced changes consistent with UV radiation damage in the canine disease, to understand the role of solar exposure on tumour development and progression. There have also been no studies so far investigating the significance of malignancy features in discerning between solar-induced and non-solar-induced cHSA via microscopic examination. This aspect of the study would aid in predicting clinical outcome in dogs and aetiology of the tumour based on microscopic malignancy features instead of survival data.

The first objective of this study is to compare the detection of solar-induced histopathological changes between canine cutaneous HA and HSA. The second objective is to determine the association between solar-induced changes with malignancy features in canine cHSA.

There are two hypotheses for this study, which includes:

- Null hypothesis 1: There is no difference in detection of solar-induced changes when comparing canine cHSA and cHA.
- Alternative hypothesis 1: There is a significantly higher detection of solar-induced changes in canine cHSA compared to cHA.

and

- Null hypothesis 2: There is no association between solar-induced changes with malignancy features in canine cHSA.
- Alternative hypothesis 2: There is an association between solar-induced changes with malignancy features in canine cHSA.

In this study, we expect to find a higher detection of solar-induced histopathological changes in canine cHSA as compared to cHA. We also expect to find an association between the solar-induced histopathological changes with malignancy features in cHSA.

2.0 LITERATURE REVIEW

2.1 Canine visceral haemangioma and haemangiosarcoma

2.1.1 Aetiology and pathogenesis

HSA is a form of cancer that develops from the vascular endothelial cells, and has recently found to be possibly originating from a pluripotent bone marrow progenitor, that are close to or at the point of differentiating into an endothelial lining (Kim et al., 2015; Gordon et al., 2014; Lamerato-Kozicki et al., 2006; Fosmire et al., 2004). The development of HSA may very likely occur because of genetic mutations in tumour suppressor genes and oncogenes (Griffin et al., 2021). Certain tumour suppressor genes such as p53, phosphatase and tensin homolog (PTEN) and Ras that has undergone mutations have been found in association with HSA (Naka et al., 1997; Garcia et al., 2000; Dickerson et al., 2005; Wang et al., 2017). Besides, HSA formation may also be attributed to abnormal regulation of angiogenesis (Griffin et al., 2021). Studies have shown elevated levels of plasma vascular endothelial growth factor (VEGF) and serum endothelin-1 in dogs with HSA compared to the control group (Clifford et al., 2001; Fukumoto et al., 2015). The important recurrent features of HSA cells include inflammation and angiogenesis, and these cells express receptors that produces relevant signals when attached to chemokines such as interleukin (IL)-8 (Gorden et al., 2014; Tamburini et al., 2010). IL-8 has been proposed to play a role in modulating tumour microenvironment, leading to the proliferation and survival of neoplastic cells (Kim et al., 2015). Overall, HSA pathogenesis remains complex and multifactorial.

2.1.2 Neoplastic behaviour and clinical manifestation

The most common primary site for development of canine HSA is the spleen (28-50%), and other primary sites include the right atrium and auricle (3-50%), and the skin and subcutaneous tissue (13%) (Hosgood, 1991; Aronsohn, 1985; Wykes et al., 1986; Ward et al., 1994). HSA usually metastasizes to the liver, mesentery, omentum, and lungs, commonly through the hematogenous route or via tumour rupture leading to seeding of neoplastic cells through a body cavity (Withrow et al., 2013; Brown et al., 1985; Waters et al., 1988). Interestingly, HSA is also the most frequently found primary neoplasm in the canine heart, and seems to be the neoplasm that typically metastasizes to the brain in dogs (Ware and Hopper, 1999; Yamamoto et al., 2013; Treggiari et al., 2017; Waters et al., 1989; Snyder et al., 2008). As compared to HSA, HA mostly develops on the skin (Hargis et al., 1992). According to Gross et al. (2005), a few HA variants reported include infiltrative, capillary and cavernous, granulation tissue-type, arteriovenous, spindle cell, angiokeratomas, solar-induced, and angiolipomas, whereas known variants of HSA include epithelioid and solar-induced.

Haemangiosarcoma is usually seen in older dogs, and there seems to be an increased risk in large breed dogs. Generally, any dog breed may develop this form of cancer. However, one of the most common dog breeds reported to develop HSA is the German shepherd dog (GSD), and it was also reported that in Golden Retrievers dying because of cancer, the most common histopathological diagnosis was HSA (Kent et al., 2018). On clinical presentation, HSA may present as a solitary mass, multifocal growth limited to an organ or a wide spread growth involving metastasis (Griffin et al., 2021). Grossly, HSAs are typically poorly circumscribed, non-encapsulated and have a degree of

friability that may possibly cause tumour rupture and haemorrhage as further complications (Withrow et al., 2013). For clinicians, a clinical staging system (Stage I, II, III) has been established for canine HSA to evaluate the size and extent of the primary tumour growth, lymph node metastasis and distant metastasis (Withrow et al., 2013).

2.1.3 Diagnosis and tumour grading

Histopathology remains as the gold standard for the definitive diagnosis of HA and HSA. However, it may be a challenge to differentiate HSA, HA and haematoma on histopathology because of the cavitary appearance of HSA, and thus larger tissue biopsies are necessary to aid in the confirmatory diagnosis of HSA (Patten et al., 2016; Herman et al., 2019). Thus, it is recommended to keep a degree of malignancy suspicion in dogs, regardless of the histopathologic diagnosis of a benign disease, due to the challenge in differentiating the tumour types and the utterly dissimilar prognosis between them (Griffin et al., 2021). Though a grading system for canine splenic HSA exist, which is based on tumour differentiation, nuclear pleomorphism, mitotic count and tumour necrosis, it is rarely used as canine HSA generally has a poor prognosis and are usually falls into the highest grade (Grade III) (Ogilvie et al., 1996; Avallone et al., 2021). A study on dogs with HSA receiving doxorubicin as adjuvant chemotherapy suggested several parameters in the histologic grading system (tumour differentiation, higher mitotic count, increased nuclear pleomorphism) as possible prognostic indicators (Ogilvie et al., 1996).

2.1.4 Treatment and outcome

Surgery remains as the mainstay and preferred initial therapy for HSA. For dogs with

splenic HSA, a total splenectomy is the best option, along with a thorough exploratory laparotomy to excise any lesions that may indicate metastatic disease (Withrow et al., 2013). Although treatment with surgery can alleviate symptoms and stop signs of haemorrhage for months in some animals, surgery alone may not be sufficient as dogs may die of metastatic disease (MacEwen, 2001). Studies have reported a median survival time ranging from 19 to 86 days in dogs with splenic HSA that have undergone splenectomy alone (Prymak et al., 1988; Wood et al., 1998; Goritz et al., 2013; Batschinski et al., 2018; Wendelburg et al., 2015). Besides surgical resection, various adjuvant chemotherapy protocols have also been described for canine HSA, with doxorubicin having the best chemotherapeutic efficacy for this disease (Smith, 2003). One study also found that epirubicin had a similar efficacy when used as adjuvant therapy for canine splenic HSA (de la Fuente et al., 2014; Brown et al., 1985; Kim et al., 2007). A significant difference in median survival times (MST) has been reported, where the MST of dogs treated with surgery alone was 66 days, while the MST of dogs treated with surgery and adjuvant chemotherapy (doxorubicin) was 274 days (Batschinski et al., 2018). However, other studies did not report a statistically significant improvement in survival time when comparing dogs with splenic HSA that were treated with surgery alone and with those treated with surgery and chemotherapy (Griffin et al., 2021).

2.2 Canine cutaneous haemangioma and haemangiosarcoma

2.2.1 Aetiology and pathogenesis

Dogs are most affected by cHSA when compared to other domesticated species. Studies in Brazil have shown that there was a high prevalence of cHSA cases of up to

27-80% of all canine HSA cases (Flores et al., 2012; Soares et al., 2017). This finding was associated with the high ultraviolet (UV) radiation levels in the tropical country (> 6.0), which was an aspect directly linked to the aetiology of cutaneous actinic HSA (DSA-INPE, De Nardi et al., 2023). Most of the immediate photobiological responses in the skin are triggered by high-energy light within the UVB range of 290-320 nm (Maxie, 2016). Lights with wavelength ranging from 320 to 400 nm are under the UVA category and may further enhance UVB-induced harm (Maxie, 2016). The skin is usually protected against the harmful effects of UV radiation via the haircoat, melanin pigmentation, and the stratum corneum (Maxie, 2016). Thus, predisposed breeds with reduced skin pigmentation and less hair would have lower protection from the radiation and a higher level of sun exposure. Inflammation of the skin and oxidative stress can occur because of acute exposure to UVB radiation, whereas carcinogenesis may occur because of chronic exposure (De Nardi et al., 2023).

Scientific papers have found that reactive oxygen species (ROS) acts as a link between chronic inflammation and tumour development. Early investigations on the influence of ROS in the initial stages of neoplasia development have suggested that oxidative stress directly harms DNA, encouraging genetic mutations that favours malignant transformation (Cezar et al., 2019; Nediani and Dinu, 2022). UV radiation can act as a complete carcinogen, a co-carcinogenic agent, or an immunological modulator, relying on the dosing plan (Roberts et al., 1986). Besides, when investigated in mice, UV radiation was found to not only be merely tumourigenic, but also alters the immune system, causing the mice to be susceptible to UV radiation associated tumours (Kripke and Fisher, 1976). There have also been studies displaying the consistent formation of

a carcinogen (cholesterol-oxide) from natural sterols in the skin of albino hairless mice and humans due to exposure to UV radiation (Lo et al., 1974). Furthermore, enzyme systems that neutralize this carcinogen have also been found (Winkelmann et al., 1963).

For the non-actinic form of cHSA, breeds other than those predisposed (pigmented skin, thicker hair coats, history of chronic sun basking) are more frequently found to develop this form of tumour. Thus, actinic changes are not detected during the histopathological evaluation of these tumours (Szivek et al., 2012). Solar exposure most likely does not play a role in the development of the non-actinic form, and although the exact aetiology is still unknown, previous studies suggests that the aetiopathogenesis is most likely reflecting that of visceral HSA (Kim et al., 2015). When genomic profiles were analysed to detect any mutations in specific genes, several studies have found that the most mutated gene in cHSA of dogs is TP53, like visceral HSA (Wong et al., 2021). A study by Garcia-Iglesias et al. (2020) discovered a likely association between TP53 gene mutation and neoplasia development when the researchers found a high level of mutated TP53 gene which was associated with elevated Ki-67 proliferative activity in dogs with cHSA (Garcia-Iglesias et al., 2020). Besides, studies on cutaneous and subcutaneous HSAs have also detected mutations in PTEN genes and a high level of factor expression such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), further suggesting their involvement in the pathogenesis of the disease (Nobrega et al., 2019; Wong et al., 2021; Rivera-Calderon et al., 2019; Yonemaru et al., 2006). However, further studies are required to confirm these suggestions.

2.2.2 Neoplastic behaviour and clinical manifestation

According to Ward et al. (1994) and Shiu et al. (2011), HSA can be mainly cutaneous, with subcutaneous and muscular infiltration, or mainly subcutaneous, with intramuscular involvement. Studies have found that the actinic form of cHSA tends to develop most frequently on the ventral abdomen, preputial region, and the pelvic limbs of predisposed canine breeds with a history of chronic sun exposure (Nobrega et al., 2019; Ward et al., 1994; Szivek et al., 2012). Generally, solar-induced cHAs are more common than solar-induced cHSAs, and may signify a transition from vascular changes to malignancy (Werner et al., 2014). Early lesions of cHA are usually small, flat, and punctate in appearance with purplish discolouration. However, with time, these lesions may increase in size to firm or fluctuant, reddish-purple plaques or nodules on the skin. As the lesions grow larger, they may ulcerate and bleed (Werner et al., 2014).

Cutaneous HSA on the other hand, is usually manifested as solitary or multifocal superficial nodular/papular growths with reddish to black discolouration. The growth is usually present locally with possible slight intermittent bleeding (Mullin and Clifford, 2020; Ward et al., 1994). Though histologically the actinic and non-actinic subtypes of cHSA have the same origin, they have different biological behaviour (De Nardi et al., 2023). It has been reported that there appears to be an increased metastasis risk and reduced survival time in dogs with dermal HSA that are non-solar induced and invasive into the subcutaneous tissue, as compared to the solar-induced and non-invasive dermal HSA (Szivek et al., 2012). However, actinic cHSAs are more likely to develop repeatedly in the same skin region over time due to a field cancerization effect. Field cancerization effect describes the recurrence of cancer development in the same region

with prolonged and continuous exposure to a carcinogen, such as UV radiation, leading to the development of multiple microscopic neoplastic foci (Szivek et al., 2012).

2.2.3 Diagnosis and staging

Histopathology remains as the gold standard diagnostic method for cHA and cHSA. Pre-surgery screening with cytology may be carried out, but it usually lacks diagnostic significance due to a high risk of haemodilution and blood contamination. For the diagnosis of cHSA, findings of a typical gross presentation of the lesions paired with the detection of malignant mesenchymal cells in cytology, are strong evidence of HSA (De Nardi et al., 2023). For the diagnosis of cutaneous HA, primary cytologic features include presence of vascular channels of different sizes lined by well-differentiated and uniform endothelial cells, where the vascular channels are held by dermal collagen fibers and collagenous septa (Stannard and Pulley, 1978). Endothelial cells with uniform nuclei that are flattened and squamoid or a little plump and ovoid, lack of mitosis and scanty cytoplasm are also standard features to look for (Hargis et al., 1992). As for cutaneous HSA, primary cytologic features include presence of vascular channels of different sizes lined by pleomorphic spindle-shaped cells that have vesicular, ovoid to elongated nuclei (Stannard and Pulley, 1978). A study has reported that in 11 cases of cutaneous and subcutaneous HSA, 100% of the cases revealed irregular vascular channels and lacunae, prominent endothelial lining with cytological atypia and newly formed unusual blood channels made of neoplastic endothelial cells within the tumour (Abramo et al., 2022).

Solar-induced changes that have been identified in cHSA lesions include solar elastosis, superficial dermal fibrosis, epidermal dysplasia, and dermatitis which will be further described in the following subsections (Szivek et al., 2012). Another study found that some of the solar-associated histopathological changes common in dogs with lightly pigmented skin and short hair coat include solar dermatitis and actinic keratosis, and dogs with a history of frequent sunbathing are more likely to develop these skin lesions (Albanese et al., 2013). Solar elastosis was another common solar-induced change observed. In one study, 18% of dogs with a total of 46 cutaneous HA and HSA showed solar elastosis next to the tumour growth in the dermis, and 13% of the said dogs also developed squamous cell carcinoma in the glabrous ventral skin (Hargis et al., 1992). A study by Ward et al. (1994) used a clinical staging in cHSA cases; Stage I: primary tumour limited to the dermis, Stage II: primary tumour involving the subcutaneous tissue, with or without concurrent dermal involvement and without muscular involvement, and Stage III: primary tumour with muscular involvement (Ward et al., 1994).

2.2.4 Treatment and outcome

Like visceral HA and HSA, the initial treatment option would be surgery, with or without adjuvant chemotherapy, depending on presence of metastatic disease. For dogs with stage I cHSA, it is recommended for surgical removal to be carried out with lateral margins of 1-2 cm and deep margins in the fascial plane (De Nardi et al., 2023). When these lesions are completely resected, dogs can be cured, especially in cases of cHSA with solar-induced changes with no indication of adjuvant chemotherapy (Ward et al., 1994; Griffin et al., 2021; Szivek et al., 2012). For dogs with more infiltrative tumours,

such as those involving the subcutaneous and muscular tissues, surgeries usually require a larger lateral margin of at least 3 cm (Mullin and Clifford, 2020; Griffin et al., 2021). However, since the non-actinic form of cHSA has a more aggressive and metastatic nature, more studies should be carried out to determine its clinical benefit (Szivek et al., 2012).

Adjuvant chemotherapy is more so indicated for subcutaneous and muscular HSA which have a more aggressive biological behaviour, and like visceral HSA, doxorubicin is also the most frequently used (Sorenmo et al., 1993; Ogilvie et al., 1996; Bulakowski et al., 2008; Hammer et al., 1991; Alvarez et al., 2013). Not only can doxorubicin be used as a single chemotherapeutic agent, it can also be used as a combination therapy. The combination of doxorubicin, dacarbazine and vincristine as a three-drug chemotherapy protocol has proven to prolong the ST in advanced stages of canine HSA (Dervisis et al., 2011). In research by Sharun et al. (2019) on cHSA, there was a prolonged ST post-surgery (10-11 months) in dogs treated with both surgical excision and adjuvant chemotherapy with doxorubicin and vincristine. It has been reported that the median overall ST in a study on canine cutaneous HSA was 365 days, meaning that dogs with cutaneous HSA have a 50% chance of surviving for more than 365 days (Nobrega et al., 2019). The median ST in dogs with dermal HSA was 780 days, while those with subcutaneous and muscular infiltration had much lower ST of 172 and 307 days, respectively (Ward et al., 1994; Nobrega et al., 2019). Generally, dogs with dermal HSA tend to have a better prognosis than those with visceral HSA. MST exceeding 2 years has been reported in dogs with dermal HSA after surgery alone

which suggests that surgery alone may be a practical treatment of choice for canine cutaneous HSA (Griffin et al., 2021; Ward et al., 1994).

2.3 Histopathology as a diagnostic method

2.3.1 Haematoxylin and eosin staining

In histologic examination, the entire tissue sample on the slide should be evaluated at low power magnification before proceeding to focus on individual histopathologic lesions and cells, to avoid misdiagnosis. Haematoxylin and eosin (HE) is the routine histologic staining carried out as it is highly effective in pathology due to the strong attraction of eosin (an acidic dye) to cytoplasmic proteins, and the affinity of haematoxylin (a basic dye) for nuclear structures (Maxie, 2016). The staining method has been maintained for many years as it works well with many different fixatives and is able to show a wide range of cytoplasmic, nuclear, and extracellular matrix structures, which forms the foundation of cancer diagnosis (Fischer et al., 2008). The staining process typically includes steps such as dewaxing, hydration, staining with haematoxylin, differentiation, staining with eosin, dehydration, clearing and cover-slipping.

2.3.2 Solar-induced histopathological changes

Solar-induced histopathologic changes can be identified in cutaneous tumours such as squamous cell carcinoma, haemangioma and haemangiosarcoma, where UV radiation exposure plays a role in tumour development and progression. Solar dermatitis, otherwise known as sunburn, encompasses histologic changes such as epidermal hyperplasia (epidermal thickening caused by increased number of keratinocytes),

epidermal dyskeratosis (abnormal epidermal maturation), actinic comedone formation (clogging of follicular pore with keratinocytes and sebum), presence of sunburn cells (apoptotic keratinocytes), sublaminar fibrosis (increased fibrous connective tissue), elastosis (wavy, thickened and tangled fiber that are more basophilic) and focal areas of actinic keratosis (Maxie, 2016; Albanese et al., 2013). Actinic keratosis may indicate a premalignant condition, and many researchers have found a close association and genetic resemblance between actinic keratosis and squamous cell carcinoma (Albanese et al., 2013). Several studies have defined actinic change by identification of solar elastosis, superficial dermal fibrosis, dermatitis, and epidermal dysplasia (Szivek et al., 2012; Garcia-Iglesias et al., 2020), while others have focused on the occurrence of solar elastosis to confirm the role of solar exposure on tumour development (Hargis et al., 1992; Ward et al., 1994). A study reported two Dalmatians with repeated exposure to the sun developing cutaneous HA associated with dermal elastosis as a solar-induced change, and the actinic change was also associated with the development of squamous cell carcinoma (Knowles and Hargis, 1986).

2.3.3 Grading and malignancy features

Tumour grading on tissue samples is the microscopic evaluation and quantification of parameters that align with the presumed clinical aggressiveness of a tumour, primarily relying on the tumour histomorphology (Avallone et al., 2021). In the case of canine HSA, parameters evaluated in the grading scheme include differentiation, nuclear pleomorphism, mitotic count and tumour necrosis, and the summation of scores would give the final tumour grade (Ogilvie et al., 1996). Elements of this grading system have been proposed as potential prognostic indicators for dogs diagnosed with HSA, and a

study also found an association between the grading system with overall survival which was displayed on univariate analysis (Moore et al., 2017; Ogilvie et al., 1996). Since to date there is still no standardized grading system for the cutaneous form of HSA, thus it becomes interesting to explore the potential of evaluating histopathology features of malignancy to correlate with survival outcomes. A study by Makinen et al. (2017) on pulmonary adenocarcinomas, investigated the correlation of classical histopathology malignancy characteristics such as nuclear atypia, mitotic activity, tumour necrosis and tumour invasiveness with outcome, and found a significant correlation. This validates the prognostic significance of the growth pattern analysis and suggests the potential of evaluating malignancy features microscopically, for pathologists to predict outcome.

3.0 MATERIALS AND METHOD

3.1 Study design and consents

Retrospective research was conducted on cases diagnosed as canine cutaneous haemangioma and haemangiosarcoma at the Histopathology Laboratory of the Faculty of Veterinary Medicine, Universiti Putra Malaysia from 2018 to 2023. Consents were first obtained from the histopathology laboratory to retrieve data from the log books and laboratory submission forms. This study proposal was approved by the Final Year Project Proposal Reviewing Committee of the Faculty of Veterinary Medicine of UPM.

3.2 Clinical data acquisition

Histopathology reports and laboratory submission forms were retrieved to obtain epidemiological data such as age, breed, sex, as well as additional information on history and specimen collection method. Based on histopathology reports and submission forms, the gross appearance of the tumour was also noted, including the anatomic site. Information that was lacking from the laboratory request forms were obtained by contacting the clinician who submitted the samples to the laboratory, which is mainly from private veterinarians.

3.3 Tumour tissue identification and selection

All formalin-fixed paraffin-embedded (FFPE) tumour tissue blocks diagnosed as HA or HSA were retrieved from the laboratory archives, and were microscopically evaluated to include only cutaneous forms of the tumour. All visceral, subcutaneous, and intramuscular forms of the tumour were excluded in this study.

3.4 Routine histopathology staining

Each cutaneous FFPE tissue was sectioned to 4µm thick for routine histopathology staining with haematoxylin and eosin (HE). The sectioned tissues were then placed into a water bath of 42℃, before being fished out onto glass slides, labelled accordingly, and left to dry. In the HE staining procedure, the slides were firstly dewaxed in xylene for 5 minutes, and then dipped into a series of decreasing alcohol concentrations (100% and 70% alcohol for 5 minutes each), to remove the xylene and for rehydration. The slides were then rinsed and stained with haematoxylin for 5 minutes, and then rinsed again before submerging into 1% acid alcohol (weak acid alcohol) for 3 seconds. The slides were then subjected to running tap water for 5 minutes before being stained with eosin for 1 minute. Next, slides were sprayed with 95% alcohol, placed in running tap water for another 5 to 10 seconds, cleaned and air dried before being mounted with a mounting medium (Micromount, Leica Biosystems, USA) with cover slips.

3.5 Microscopic evaluation

All the slides were then assessed using a light microscope (Eclipse Ci-L, Nikon, Japan) with a camera control unit (DS-U3, Nikon, Japan) and a camera head (DS-Fi1, Nikon, Japan) for solar-induced changes. Specific solar-induced changes evaluated included sunburn cells, solar elastosis, dermal fibrosis, and actinic keratosis (Szivek et al., 2012; Maxie, 2016; Albanese et al., 2013). Both cHA and cHSA samples were then classified into those with actinic change and without actinic change based on the above (Szivek et al., 2012; De Nardi et al., 2023). Other non-specific solar induced changes evaluated included comedone formation, epidermal hyperplasia, hair follicle infundibular dilatation

and telangiectasia (McHale and Banovic, 2022; Millanta, 2022; Poggiani et al., 2012). Degree of inflammatory infiltrates (absent, mild, moderate) and the depth of invasion (limited to dermis and invaded into the hypodermis) were determined. The cHSA samples were then graded according to the canine HSA grading scheme by Ogilvie et al. (1996), which was proposed to be for both visceral and non-visceral HSA. In the grading system, features including differentiation, nuclear pleomorphism, mitotic count in 10 high power [×40 objective] fields in 2.37 mm2, and tumour necrosis were evaluated. Microscopic images were obtained using an imaging software (NIS-Elements F 4.60.00 64-bit, Nikon, Japan).

3.6 Statistical Analysis

Statistical analysis was performed using SPSS version 29.0, and data tabulation, pie and bar charts were created using Microsoft Excel. All statistical data were tested for normality as needed using the Shapiro-Wilk test, where a p-value of >0.05 displayed a normal distribution of data. Descriptive statistics were carried out on data regarding dog signalment (age, sex, breed), and tumour site. Fisher's exact test was performed to investigate for association between nominal variables (solar-induced changes, depth of invasion) and the tumour types. Mann-Whitney U test was carried out to evaluate for differences between ordinal variables (inflammatory infiltrates, mitotic count). Comparison between solar-induced changes and malignancy features were also carried out using Fisher's exact test and Mann-Whitney U test accordingly. Statistical analysis was considered significant at a P value < 0.05.

4.0 RESULTS

4.1 Animals

There was a total of 30 cases identified from the archives with 10 diagnosed as cHA and 20 diagnosed as cHSA. There were more cases of cHSA diagnosed as compared to cHA. By tumor type, cHA had the range of age of 3 to 15 years (for 9 cases with data available for age) with a mean (± standard deviation) of 9.1 ± 3.8 years, while cHSA had the range of 3 to 14 years (for 16 cases with data available for age) with a mean (± standard deviation) of 9.4 years ± 2.5 years.

Among the dogs with cHA, 50% were females, 30% were males and 20% of the cases lacked this information. For the canine cHSA tumours, 60% were females, 35% were males and 5% with missing data. Overall, cases were more frequent in females. However, there was no significant association found between sexes and the tumour types upon running the Fisher's exact test.

Most of the cHA cases involve pedigree breeds (80%, n = 8), one mixed breed dog (10%, n = 1), and another dog with missing information for breed (10%, n = 1). Among the pedigree breeds, each case was represented with a different breed which include the Poodle, Shih Tzu, Lhasa Apso, Great Dane, Cocker Spaniel, Bulldog, Malinois and Miniature Schnauzer; however, cHSA cases were most frequently presented by mixed breed dogs (50%, n = 10), and less by pedigree breeds (25%, n = 5) with 5 other dogs with missing information for breed type (25%, n = 5). Among the pedigree breeds, there were two cases involving Pitbulls, and one case each in a Beagle, Shih Tzu and Jack Russel Terrier.

As for tumour site distribution, many cHA developed on the limbs (60%, n = 6), followed by the neck (20%, n = 20), and multifocal growth (20%, n = 20%) (Fig. 1). The majority of cHSA tumours were condensed at the ventral abdomen (55%, n = 11). Among the growths at the ventral abdomen, most cases had tumour growth directly at the ventral abdomen (20%, n = 4), followed by the inguinal region (15%, n = 3), male genitals (15%, n = 3), and mammary gland (5%, n = 1). Other locations involved were multifocal areas (20%, n = 4), limbs (15%, n = 3), thorax region (5%, n = 1), and the tail (5%, n = 1).

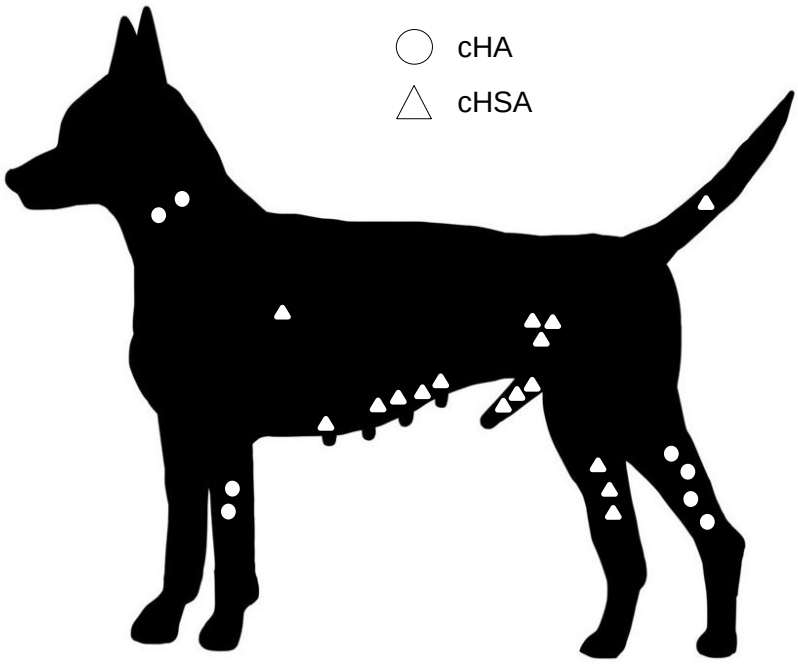

Fig 1. Anatomical site distribution of cutaneous haemangioma and cutaneous haemangiosarcoma growth. (multifocal growths are not included in this figure)

4.2 Histopathological diagnosis of cHA and cHSA

In cHA cases, the mass was confined to the dermis and elevated the overlying epidermis. cHAs were characterized by the presence of uniform blood-filled vascular channels of various sizes, lined by a single layer of well-differentiated, matured and flattened endothelial cells supported by thin collagenous septa (Fig. 2a, 2b). The endothelial cells had flattened nuclei with scanty cytoplasm with minimal to rare evidence of mitosis (Fig. 2c). cHSA was diagnosed histologically when masses contain poorly differentiated vascular channels, and increased cellular pleomorphism. These vascular channels took the shape of slit-like spaces filled with erythrocytes (Fig. 2e). Contrary to the benign growth, the neoplastic endothelial cells were plump and prominent, with moderate to marked anisocytosis and anisokaryosis. There were occasional piling-up of the endothelial cells, and in some cases, neoplastic cells display a “hobnail” appearance (Fig. 2f). Some cases were characterized by sheets of pleomorphic spindle-like cells that appear to be intersecting and anastomosing with each other. Other features of malignancy identified were high cellularity (Fig. 2d) and frequent mitotic figures. In two cHSA tumours, there was focal area that resembled cHA. Among the cHA, epidermal ulceration was observed in 20% of tumours (n = 2), while thrombus formation was observed in 10% of cases (n = 1). Among the cHSA, epidermal ulceration was observed in 65% of the tumours (n = 13), while thrombus formation was observed in 25% of tumours (n = 5).

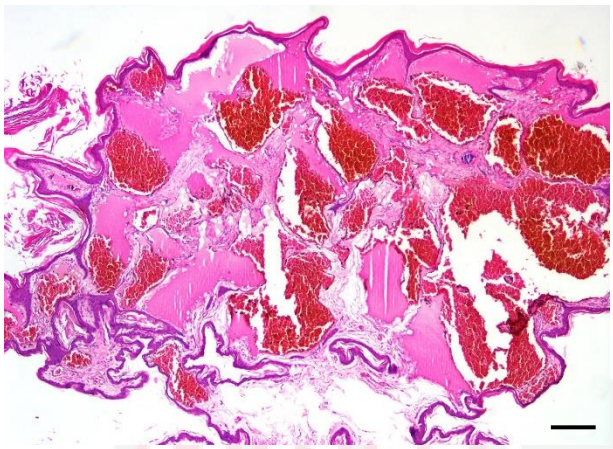

Fig. 2a

Fig. 2b

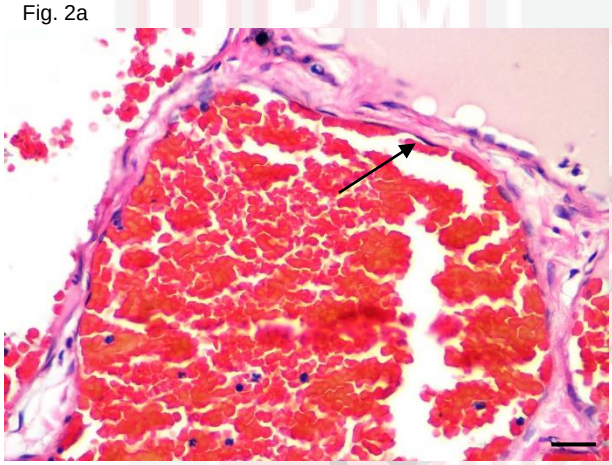

Fig. 2c

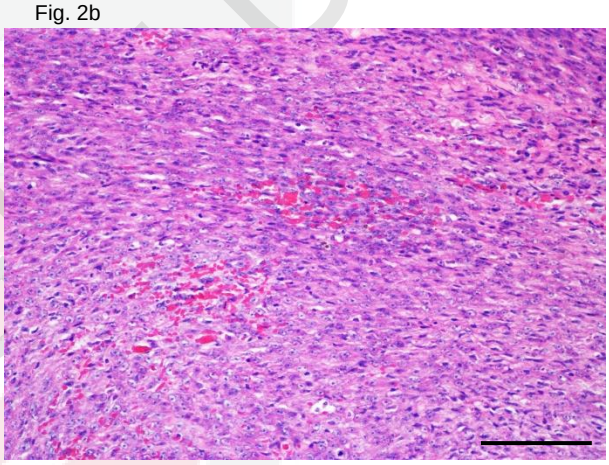

Fig. 2d

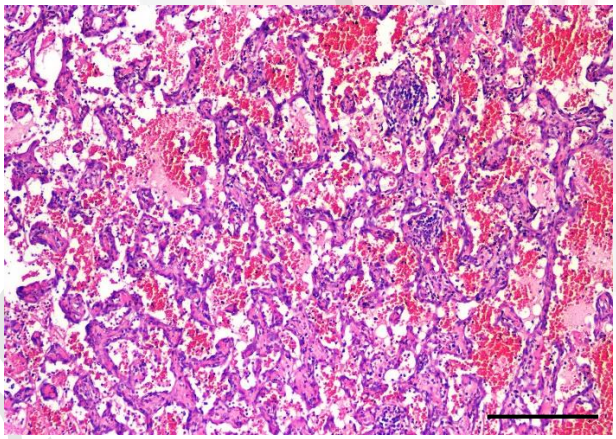

Fig. 2e

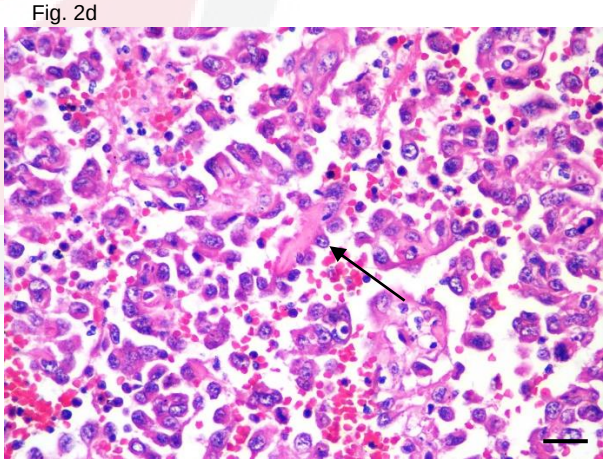

Fig. 2f

Fig. 2a. Cutaneous haemangioma. The exophytic mass is composed of uniform blood-filled vascular channels. HE, 4x Obj. Bar, 100µm.
Fig. 2b. Cutaneous haemangioma. Attenuated, flattened, well-differentiated endothelial cells line the vascular channels. HE, 20x Obj. Bar, 50µm.
Fig. 2c. Cutaneous haemangioma. Flattened, well-differentiated endothelial cells (arrow). HE, 40x Obj. Bar, 10µm.
Fig. 2d. Cutaneous haemangiosarcoma. High cellularity of neoplastic endothelial cells. HE, 20x Obj. Bar, 50µm.
Fig. 2e. Cutaneous haemangiosarcoma. Irregular, slit-like vascular channels filled with erythrocytes. HE, 10x Obj. Bar, 100µm.
Fig. 2f. Cutaneous haemangiosarcoma. The irregular slit-like channels are lined by plump endothelial cells that display a “hobnail appearance” (arrow). HE, 40x Obj. Bar, 10µm.

4.3 Solar-induced histopathological changes

Microscopically, solar-induced changes were observed in both cHA and cHSA tumours, and these changes were classified into specific solar-induced changes (changes caused by solar exposure) and non-specific solar-induced changes (changes that may be caused by other factors besides solar exposure). In cHA tumours, the only specific solar-induced changes identified was dermal fibrosis (10%, n = 1). The other specific changes such as presence of sunburn cells, solar elastosis and actinic keratosis were not identified in cHA tumours. In cHSA cases, specific solar-induced changes identified include the solar elastosis (20%, n = 4) (Fig. 3a), actinic keratosis (25%, n = 5) (Fig. 3c, 3d), and dermal fibrosis (50%, n = 10) (Fig. 3b). Presence of sunburn cells was also not found in cHSA tumours. There was a significantly higher detection of dermal fibrosis in cHSA as compared to cHA (P = 0.049), while the other changes were found to be not significant based on Fisher's exact test (Table 1). Based on the specific changes, 10% and 60% of cHA and cHSA cases respectively, had actinic changes. Within the 60% of cHSA tumours with actinic change, 83.33% of the tumour were distributed at the ventral abdomen.

The non-specific solar-induced changes assessed included comedone formation, telangiectasia, hair follicle infundibular dilatation and epidermal hyperplasia (Table 2). In cHA tumours, non-specific changes identified included hair follicle infundibular dilatation (30%, n = 3), and epidermal hyperplasia (30%, n = 3). Comedone formation and telangiectasia were not observed in cHA. In cHSA, non-specific changes identified include telangiectasia (15%, n = 3) (Fig. 4b, 4c), hair follicle infundibular dilatation (40%, n = 8) (Fig. 4a), and epidermal hyperplasia (70%, n = 14) (Fig. 4d). Comedone

formation was also not observed in cHSA. No significant association was found between the histopathological changes with tumour types. Among the cHA, there were seven tumours with absence of inflammation (70%), two with presence of mild inflammation (20%) and one tumour with moderate inflammation (10%). Inflammation was present in all cHSA tumours, where majority had mild inflammation (60%, n = 12) and others with moderate inflammation (40%, n = 8). Upon running the Mann-Whitney U test, there was a significantly higher detection of inflammatory infiltrates in cHSA as compared to cHA (P = <0.001).

Table 1. Comparison of specific solar-induced changes in cutaneous haemangioma and haemangiosarcoma

Parameter	**Cutaneous haemangioma, n = 10 (n, %)**	**Cutaneous haemangiosarcoma, n = 20 (n, %)**	**Pearson Chi Square Value**	**P value (Fisher's exact test)**
Sunburn cells	n = 0, 0%	n = 0, 0%	Not applicable	Not applicable
Solar elastosis	n = 0, 0%	n = 4, 20%	2.308	0.272
Actinic keratosis	n = 0, 0%	n = 5, 25%	3.000	0.140
Dermal fibrosis	n = 1, 10%	n = 10, 50%	4.593	0.049

Table 2. Comparison of non-specific solar-induced changes in cutaneous haemangioma and haemangiosarcoma

Parameter	**Cutaneous haemangioma, n = 10 (n, %)**	**Cutaneous haemangiosarcoma, n = 20 (n, %)**	**Pearson Chi Square Value**	**P value (Fisher's exact test)**
Comedone formation	n = 0, 0%	n = 0, 0%	Not applicable	Not applicable
Telangiectasia	n = 0, 0%	n = 3, 15%	1.667	0.532
Hair follicle dilatation	n = 3, 30%	n = 8, 40%	0.287	0.702
Epidermal hyperplasia	n = 3, 30%	n = 14, 70%	4.344	0.056

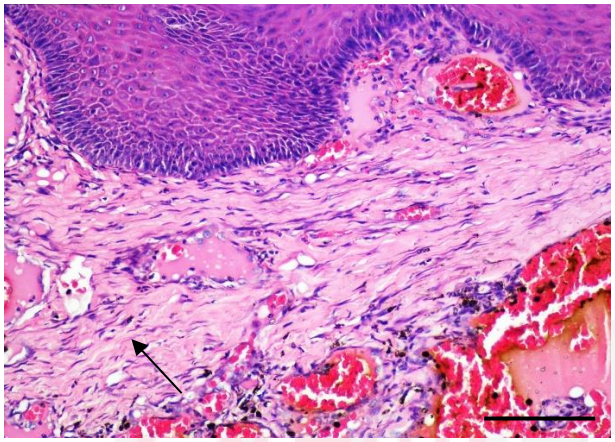

Fig. 3a

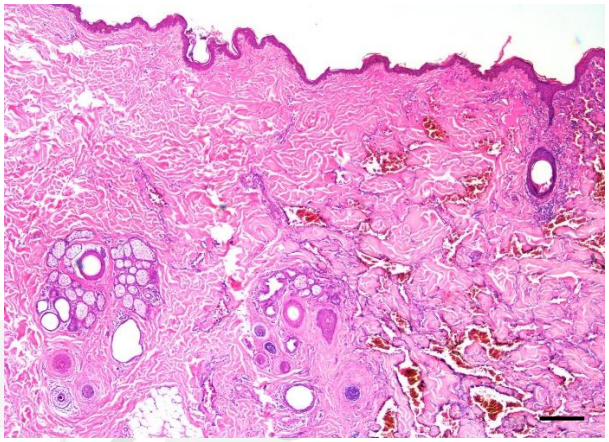

Fig. 3b

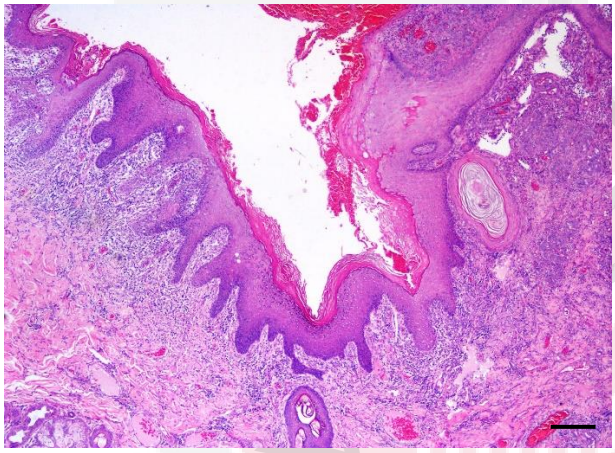

Fig. 3c

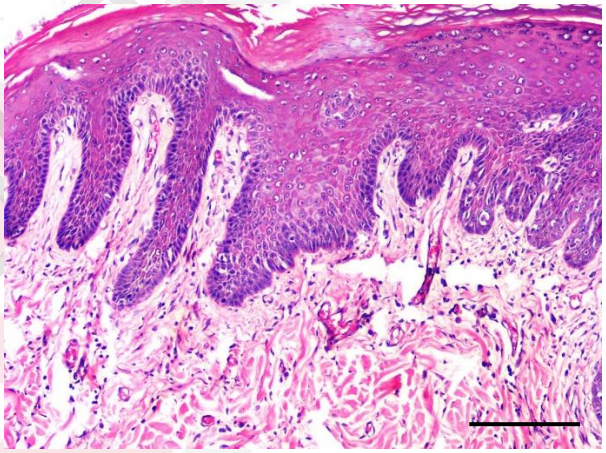

Fig. 3d

Fig. 3a. Cutaneous haemangiosarcoma. Solar elastosis (arrow) characterized by wavy, thickened and deeply basophilic fibers. HE, 20x Obj. Bar, 50µm.
Fig. 3b. Cutaneous haemangiosarcoma. Dermal fibrosis.
HE, 4x Obj. Bar, 100µm.
Fig. 3c. Cutaneous haemangiosarcoma. Actinic keratosis.
HE, 4x Obj. Bar, 100µm.
Fig. 3d. Cutaneous haemangiosarcoma. Higher magnification of actinic keratosis characterized by irregular epidermal hyperplasia with formation of rete ridges. HE, 10x Obj. Bar, 100µm.

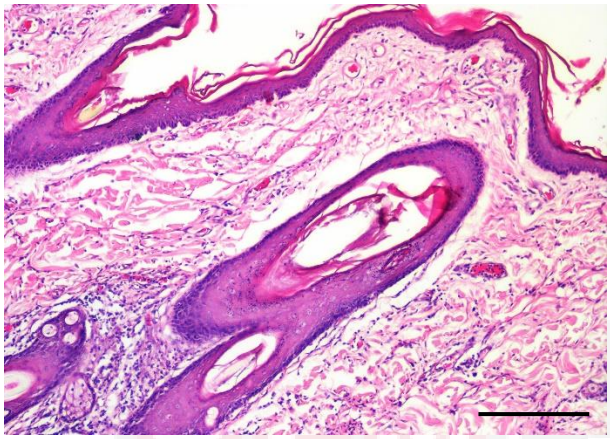

Fig. 4a

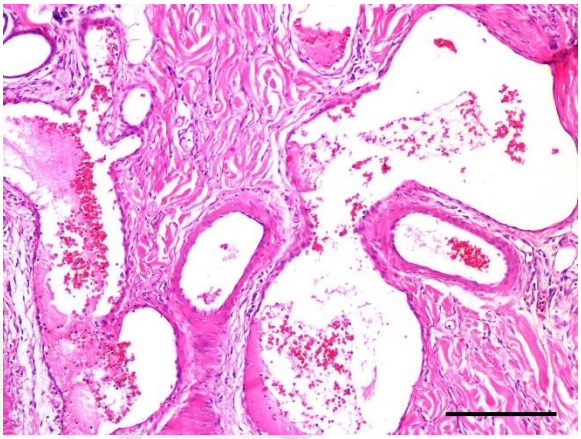

Fig. 4b

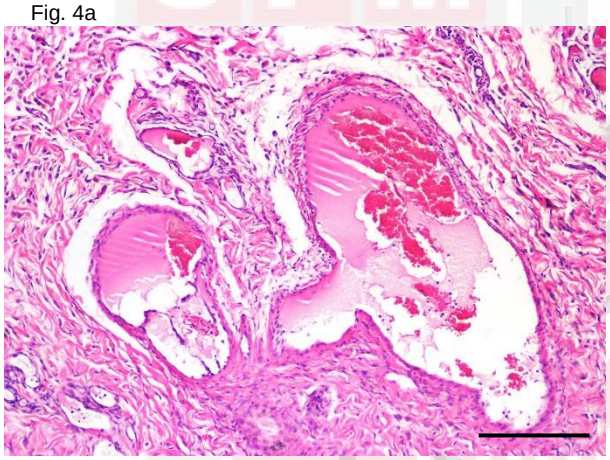

Fig. 4c

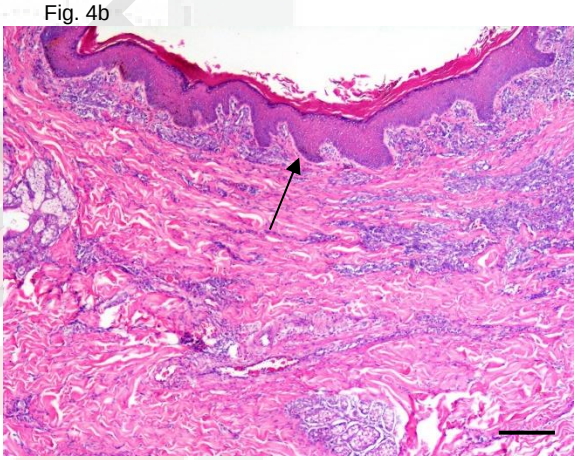

Fig. 4d

Fig. 4a. Cutaneous haemangiosarcoma. Hair follicle infundibular dilatation.
HE, 10x Obj. Bar, 100µm.
Fig. 4b. Cutaneous haemangiosarcoma. Telangiectasia.
HE, 10x Obj. Bar, 100µm.
Fig. 4c. Cutaneous haemangiosarcoma. Telangiectasia.
HE, 10x Obj. Bar, 100µm.
Fig. 4d. Cutaneous haemangiosarcoma. Epidermal hyperplasia (arrow).
HE, 4x Obj. Bar, 100µm.

4.4 Histopathological grading and malignancy features

All cHSA tumours were graded according to degree of differentiation (Fig. 5c, 5d), nuclear pleomorphism (Fig. 5e, 5f), mitotic count (Fig. 5i, 5j) and degree of tumour necrosis (Fig. 5g, 5h), and mostly were of grade I (65%, n = 13), and the rest were of grade II (35%, n = 7). As for mitotic figures, none were identified in all cHA tumours, whereas a range of 0 to 26 counts in 10 high power [×40 objective] fields were identified in cHSA. Within cHSA tumours, mitotic count ranges from those with absent mitotic figures (25%, n = 5), 1 to 10 MC (40%, n = 8), 11 to 20 MC (15%, n = 3), and 21 to 30 MC (20%, n = 4). Upon running the Mann-Whitney U test, cHSA had significantly higher mitotic count as compared to cHA (P = <0.001). Majority of the cHA were limited to the dermis (90%, n = 9), and only one tumour had invasion into the hypodermis (10%, n = 1). In cHSA, many tumours had invasion into the hypodermis (65%, n = 13), while the rest were limited to the dermis (35%, n = 7) (Fig. 5a, 5b). Upon running the Fisher's exact test, there was a significant association between depth of invasion and tumour type, where a higher proportion of invasion into the hypodermis was found in cHSA.

The cHSA tumours with actinic changes (60%, n = 12) were compared with those without the actinic change (40%, n = 8), against prognostic features such the tumour grades and the depth of tumour cell invasion. There was no significant association between the malignant tumour subtypes with actinic change and as well as the same scenario for depth of cell invasion and tumour grade. There was no significant difference between tumour grades with degree of differentiation, nuclear pleomorphism, and mitotic count in cHSA tumours with and without actinic change.

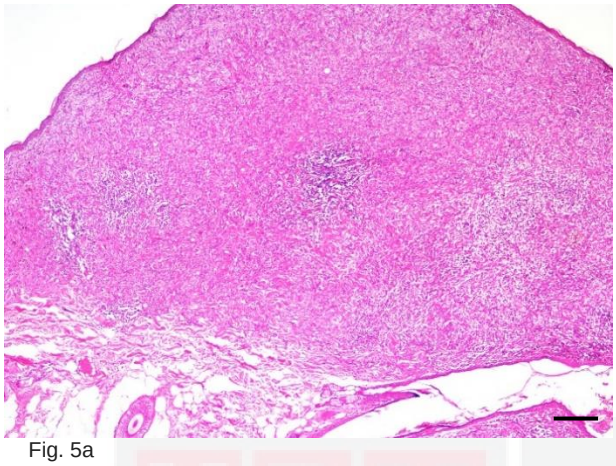

Fig. 5a

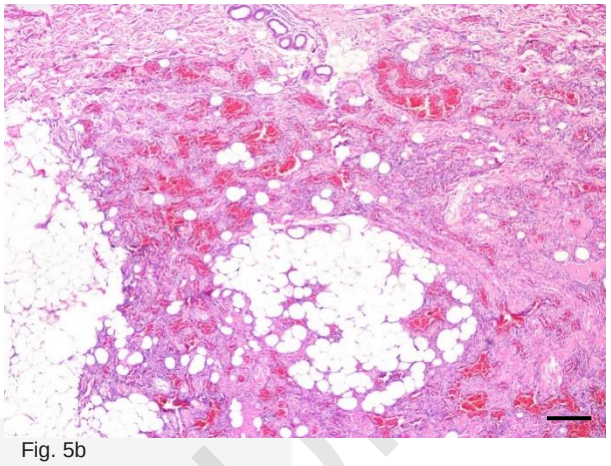

Fig. 5b

Fig. 5a. Cutaneous haemangiosarcoma. Mass limited to the dermis. HE, 4x Obj. Bar, 100µm.
Fig. 5b. Cutaneous haemangiosarcoma. Mass invaded into the hypodermis. HE, 4x Obj. Bar, 100µm.

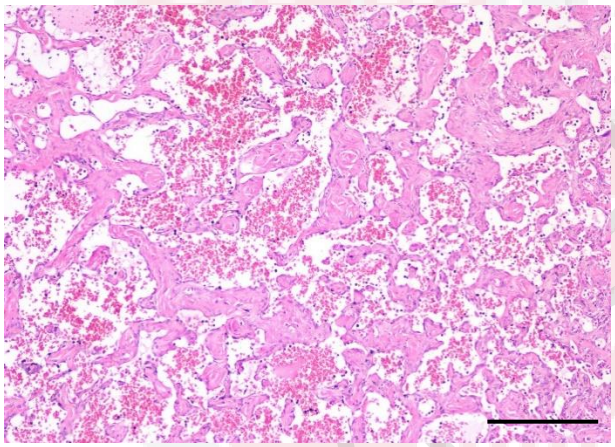

Fig. 5c

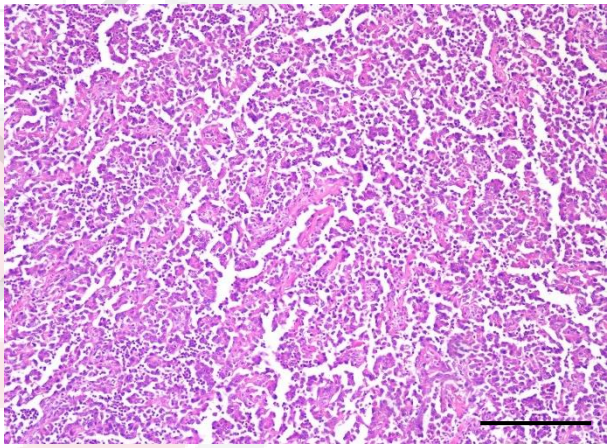

Fig. 5d

Fig. 5c. Cutaneous haemangiosarcoma. Well differentiated. HE, 10x Obj. Bar, 100µm.
Fig. 5d. Cutaneous haemangiosarcoma. Poorly differentiated. HE, 10x Obj. Bar, 100µm.

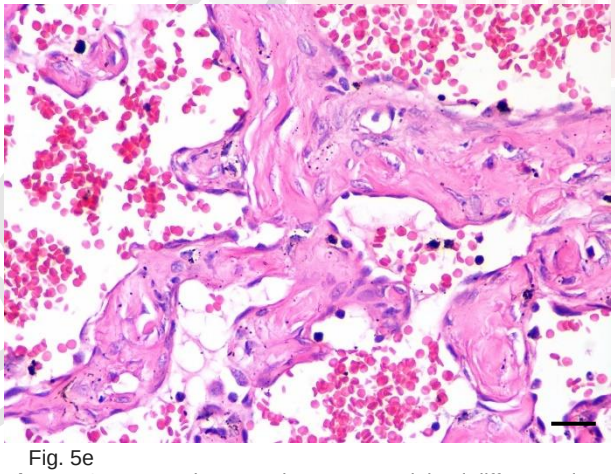

Fig. 5e

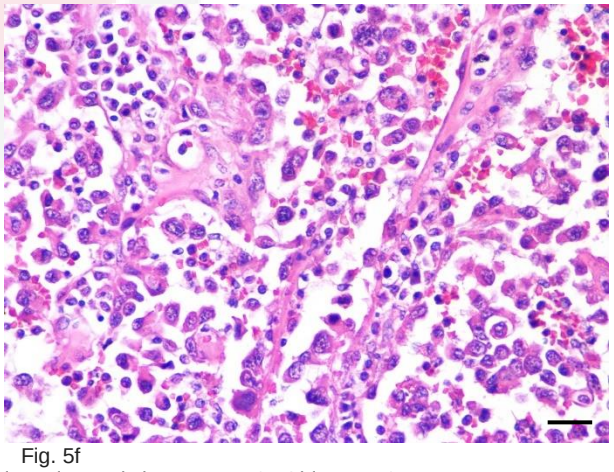

Fig. 5f

Fig. 5e. Cutaneous haemangiosarcoma. Minimal difference in nuclear size and shape. HE, 40x Obj. Bar, 10µm.
Fig. 5f. Cutaneous haemangiosarcoma. Marked variation in nuclear size and shape, >2x size difference. HE, 40x Obj. Bar, 10µm.

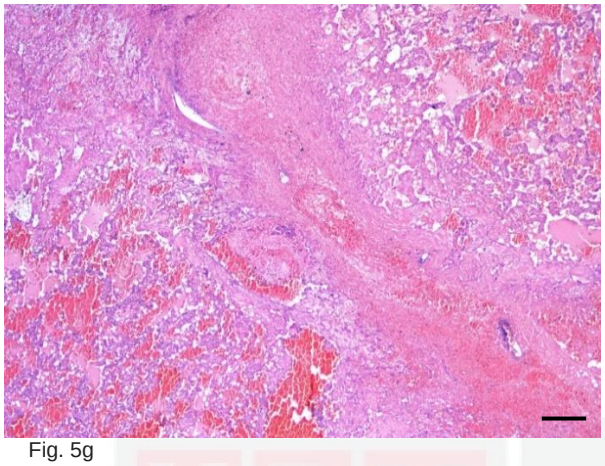

Fig. 5g

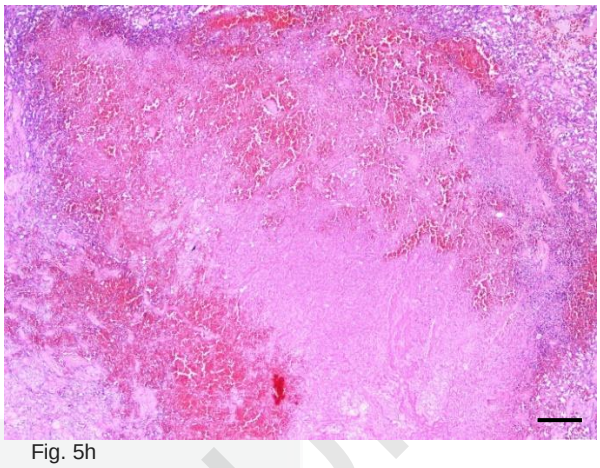

Fig. 5h

Fig. 5g. Cutaneous haemangiosarcoma. Mild necrosis, <25%. HE, 4x Obj. Bar, 100µm.
Fig. 5h. Cutaneous haemangiosarcoma. Marked necrosis, >50%. HE, 4x Obj. Bar, 100µm.

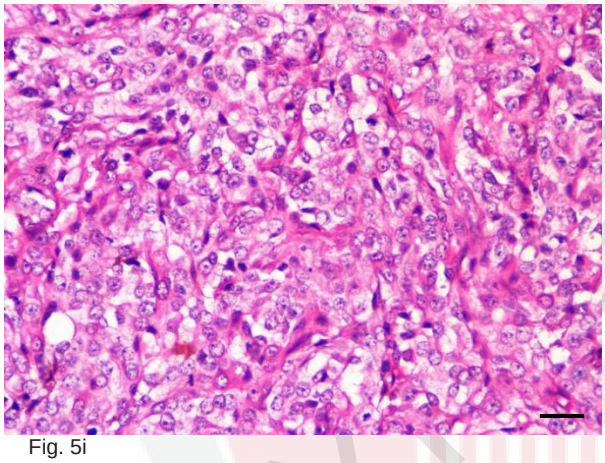

Fig. 5i

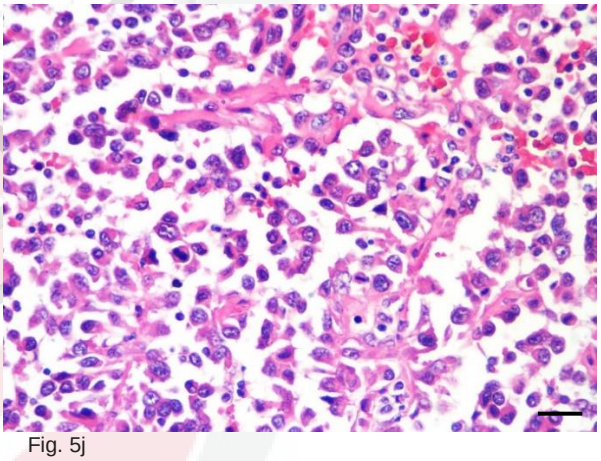

Fig. 5j

Fig. 5i. Cutaneous haemangiosarcoma. Absence of mitotic figures. HE, 40x Obj. Bar, 10µm.
Fig. 5j. Cutaneous haemangiosarcoma. Numerous mitotic figures (arrow). HE, 40x Obj. Bar, 10µm.

5.0 DISCUSSION

In this study, examining cases from 2018 to 2023, there were two times more cases of cHSA presented as compared to cHA. This finding is in contrast to previous literature which states that cHAs are more commonly reported than cHSAs (Stannard and Pulley, 1978; Werner, 2014). cHSA could be overrepresented due to the fact that dogs with more aggressive looking growths, as in cHSA, are more likely to be brought in for a check-up, and subsequently surgically removed for histopathologic examination as compared to those with more benign looking lesions that may not affect the dog as much systemically. Epidemiological information regarding age of dogs and lack of sex association were in alignment with previous studies on cHA and cHSA (Hargis et al., 1992; Ward et al., 1994; Sabattini and Bettini, 2009; Szivek et al., 2011; Nobrega et al., 2019). However, it is important to be cautious of the relatively small population size studied when interpreting the findings.

The breed distribution in cHSA cases were consistent with previous study by Nobrega et al. (2019), which reported cHSAs growths most frequently in mixed breed dogs, followed by pitbulls and other breeds. In contrast, several other studies reported cHSA most commonly in Whippets and pitbulls, which are predisposed breeds to this tumour growth (Hargis et al., 1992; Szivek et al., 2011). Short hair coats in these predisposed breeds allow for more UV radiation to reach the skin, and other factors affecting the exposure include the amount of time spent outside kennels (Nikula et al., 1992). In Malaysia, mixed breed dogs or local dogs are commonly housed outdoors within

housing compounds, possibly increasing their exposure to sunlight, which may explain the higher frequency of cases seen in this group of dogs, assuming the involvement of UV radiation in tumour development.

Majority of tumour growths, both cHA and cHSA, developed most frequently on the skin involving the ventral region of the body (ventral abdomen, inguinal region, male genitals, limbs). This finding agrees with a study carried out on beagles on the effects of ionizing radiation on incidence of skin tumour and solar dermatosis, which found the lower abdominal skin and prepuce region to be most affected (Nikula et al., 1992). Other studies on cHSA also reported similar findings due to reduced protection from solar radiation resulting from lack of pigmentation and hair growth (Ward et al., 1994; Nobrega et al., 2019). The higher occurrence of cHA and cHSA in the ventrum of dogs may also reflect the reality that due to lesser hair in these regions, identification of vascular tumour growths by owners may be easier (Hargis et al., 1992).

In this study, dermal fibrosis was a significant and notable specific solar-induced change in differentiating cHSA from cHA. Although histopathological changes of solar elastosis and actinic keratosis were not found to be significant in differentiating between tumour types, these changes were only identified in the malignant form of the tumour, which is worth noting. Dermal fibrosis, actinic keratosis and solar elastosis are solar-associated changes included under the umbrella term of solar dermatitis, which provides histologic evidence of chronically sun damaged skin that may further progress

into tumour development (Szivek et al., 2011; Albanese et al., 2013; McHale and Banovic, 2022). The findings of solar changes in this study further confirms that solar radiation has a role in the development of cHA and cHSA, and that there may be a progression of tumour growth from benign to malignant neoplasm with chronic sun exposure. Further evidence of malignant tumour progression can be extracted from this current study, where two cases of cHSA also had histologic evidence of cHA. A study on 212 dogs with cHA and cHSA found similar evidence, where HSAs were arising from preexisting HAs in sun-exposed skin (Hargis et al., 1992).

The reason as to why dermal fibrosis was the only notable change is unclear. However, a study on the response of keratinocytes and fibroblast to solar exposure found that these cells exposed to UVA and UVB had formation of cyclobutene pyrimidine dimers (CPDs), in which its accumulation was used to assess DNA damage of the cells, confirming the DNA damaging potential of UVA and UVB (Goyeneche et al., 2020). Actinic keratosis has been widely studied as a premalignant change preceding the development of squamous cell carcinoma (SCC), where presence of actinic keratosis and solar elastosis concurrently in the epidermis and dermis gives further evidence of UV radiation being part of the aetiology (Teifke and Löhr, 1996). Terrestrial solar radiation includes both UVB (290-320nm) and UVA (320-400nm) radiation (Lawrence et al., 2018). UVA radiations are the most prevalent out of all other solar UV radiation, representing about 90% of rays that touch the ground. Thus, UVA rays has a major contribution to solar damage on the skin (Millanta et al., 2022). UVB rays reach the

ground in low amounts although are highly energetic. Most of the UVB radiation is absorbed by the epidermis (70% from the stratum corneum), while only about 10% penetrates the dermis. In contrast, UVA radiation is only partially absorbed upon reaching the epidermis, while 20-30% of the rays, pierce through deeper than UVB rays. Thus, it can be concluded that UVB rays have greater effect on the epidermis compared to UVA, due to the distinction in absorption components (Battie et al., 2014). Due to this factor, UVB radiation primarily contributes to the development of squamous cell carcinomas and actinic keratoses as a premalignant lesion, while UVA induces carcinogenic effects of solar exposure by causing significant changes in the dermal connective tissue (Battie et al., 2014; De Gruijl, 2000; Millanta et al., 2022).

Besides dermal fibrosis and actinic keratosis, solar elastosis, which is referred to as the hallmark degenerative change of the dermis in humans exposed chronically to the sun, this change was identified in only 20% of cHSA cases (Maxie, 2016). This finding is consistent with a previous study that found that solar elastosis was not identified in all dogs with glabrous and lightly pigmented skin that developed cutaneous vascular tumours as solar elastosis is not constantly present in dog skin with sun-induced damage (Hargis et al., 1977). Even in human skin, solar elastosis takes up to years to develop as a visible histologic change (Lever and Schaumburg-Lever, 1983). Several studies also reported that solar elastosis is seldom found in dogs (Hargis et al., 1977; Hargis et al., 1978; Madewell et al., 1981). A study by Hargis et al. (1977) reported that solar elastosis was not identified in dogs that had lifetime exposure to the sun, albeit the numerous developments of squamous cell carcinomas in nonpigmented skin.

Despite that, solar elastosis has been a solar-associated change that was focused on in many previous researches to indicate UV radiation involvement in the pathogenesis of tumour growth, most likely because it is the characteristic degenerative alteration in human skin (Jubb, Kennedy Palmers; Hargis et al., 1992; Nikula et al., 1992; Ward et al., 1994).

Interestingly, the presence of sunburn cells or apoptotic keratinocytes were not identified in any of the samples in this study. According to Maxie (2016), sunburn cells are a characteristic microscopic feature of acute damage in sun-exposed epithelium, which may be induced as quick as within 30 minutes of sun exposure. Besides, research on mice have found that UVB radiation-induced sunburn cells were subsequently substituted by hyperproliferative keratinocytes, which led to the occurrence of epidermal hyperplasia. This suggest that both apoptosis and hyperplasia were related to one another (Ouhtit et al., 2000). This may explain the absence of apoptotic keratinocytes in the current study, further suggesting that dogs with histological changes of actinic keratosis and epidermal hyperplasia may have chronic sun exposure. Based on these specific solar-induced changes, majority of the cHSA cases (60%), had actinic change. This finding is consistent with previous study that found majority of dermal HSAs had actinic solar changes (70%), while the remaining cases (30%) had no detectable change on histopathological examination (Szivek et al., 2011). The common location of the ventrum for solar-induced malignancies in this study also matches with previous study (Szivek et al., 2011). These results could be attributed to the fact that ventral parts of the body are usually non-

pigmented with lightly haired skin, allowing more UVR to reach the skin, as well as dogs' tendency to lay on their back and bask in the sun (Nikula et al., 1992).

Non-specific solar-induced changes were not found to be significant changes that differentiated cHA from cHSA. Among these changes, only 30% of cHA had changes of epidermal hyperplasia and hair follicle infundibular dilatation, where as in cHSA, there was also relatively minimal changes observed which included 15% of tumours with telangiectasia and 40% with hair follicle infundibular dilatation. Epidermal hyperplasia however, was found in the majority of the cHSA tumours, with the p-value nearly approaching significance ($p = 0.056$). Significance may possibly be achieved with a higher cohort of tumours. In contrast, previous studies that focused on dogs with chronic solar damage have found changes such as actinic comedone and hair follicle infundibular dilatation (Albanese et al., 2013; Poggiani et al., 2012). The low observation of these non-specific changes in the current study may be due to small-sized cutaneous tissue samples sent for histopathological examination, where complete and thorough assessment of solar-induced changes may be a challenge.

The significantly higher detection of inflammatory infiltrates in cHSA compared to cHA in this study is an expected finding in agreement with previous studies that found where besides angiogenesis, chronic inflammation also plays a crucial role in the growth and progression of vascular tumours such as HSA (Movilla et al., 2017; Lashnits et al., 2020; Lashnites et al., 2021). Previous studies have found many mononuclear cells and

neutrophil polymorphs in UV-irradiated mouse skin focally in the dermis after 48 hours of UV exposure when compared to the control group (Ouhtit et al., 2000). UVR exposure induces DNA and extracellular matrix (ECM) damage in the skin, which disrupts homeostasis, leading to cellular stresses that stimulate an inflammatory state of the skin (Salminen et al., 2022). In a study on five dogs with solar dermatitis/actinic keratosis, immunohistochemical results displayed COX-2 expression in all cases (COX-2 as key mediator in inflammatory response), where subsequent treatment with firocoxib (a COX-2 selective inhibitor) led to reduced COX-2 expression and improvement of epidermal lesions (reduced hyperplastic and dyskeratotic alterations) upon histological examination on day 50 and 180 (Albanese et al., 2013).

Histopathology grading systems measure parameters that correlate with aggressiveness of clinical neoplasms, and is a widely used system in predicting tumour behaviour. However, the grading system should be assessed alongside other prognostic features, rather than as a single definitive prognostic variable (Avallone et al., 2021). In the present study, majority of the cHSA cases were of grade I, followed by grade II, with no tumours of grade III. This suggests the better outcome of cHSA when compared to the visceral form, which are generally not graded as they tend carry a poor prognosis and fall into the highest grade (Avallone et al., 2021). Within the grading scheme, the significantly higher MC in cHSA cases in this study is consistent with previous study that found no mitotic figure in dogs with cHA, while dogs with cHSA had MC ranging from 5 to 72 mitoses in 10 high-power fields (400x) (Garcia-Iglesias et al., 2020). Besides that, the higher proportion of neoplastic cell invasion into the

hypodermis found in cHSA, is in alignment with previous literature mentioning that invasion into hypodermis have been reported to be associated with increased risk of metastasis which may support our findings since metastasis is a feature of malignancy in HSA (Szivek et al., 2011).

Actinic cHSA tends to manifest in a less aggressive course, with lower probability of metastasis and longer survival time, in contrast to the non-actinic subtype (Nobrega et al., 2019; Szivek et al., 2012). Majority of cases of dermal HSA are caused by UVR exposure and damage, which represent the actinic subtype of the tumour (Szivek et al., 2011). Limitation to the dermis allows for a more proper and complete surgical excision of the mass, which may explain the lower rate of metastasis and subsequently better prognosis. Thus, in this study, there was an attempt to find some evidence of association between solar-induced histopathological changes and microscopical malignancy features, instead of survival data. Features in the grading system and other prognostic features (depth of invasion) were used instead as the parameters to correlate with neoplasm aggressiveness. However, there was no significant association found between malignancy features and solar induced changes. This may be due to the relatively smaller sample size and studies have also found that associating actinic changes with cHSA outcome for prognostic value remains as a topic of debate (Szivek et al., 2012; Schultheiss, 2004). The limitations of this study include having small-sized tissue samples, which poses a challenge to fully evaluate the histopathological solar-induced changes. Besides, the lack of clinical and survival data was another limitation in the retrospective nature of study. It would be recommended to collect more biopsy

samples per patient during surgical excision, especially growths from different anatomical locations when cHA or cHSA is suspected. Future studies should also include clinical follow-up data on prognosis and survival time to find more possible associations. Immunohistochemical studies should also be considered, such as using COX-2 markers to study its expression in cHA versus cHSA, which may provide meaningful results for the potential use of COX-2 inhibitors as adjuvant therapy in this neoplasm.

6.0 CONCLUSION

In conclusion, dermal fibrosis was detected to be significantly higher in cHSA as compared to cHA, as a specific histopathological solar-induced change. This finding shows that the first null hypothesis is rejected and is consistent with the alternative hypothesis, stating that there is a significantly higher detection of solar-induced change in cHSA cases. Besides dermal fibrosis, degree of inflammation, MC and depth of invasion were also found to be significant in differentiating between cHA and cHSA. There was no significant association found between solar-induced histopathological changes and malignancy features in cHSA, hence, the second null hypothesis is accepted.

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