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ORIGINAL RESEARCH PAPER

Serum angiopoietin-2 levels in dogs with splenic haemangiosarcoma, haemangioma, and splenitis

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ABSTRACT

Haemangioma (HA) and haemangiosarcoma (HSA) are among the most common splenic neoplasms in dogs. The survival time in splenic HSA is short, probably due to the lack of proper biological markers allowing early detection. We investigated the serum angiopoietin-2 (Ang-2) concentrations in 9 healthy dogs and 40 dogs with abnormal splenic masses. The Ang-2 concentration differences were further compared in healthy dogs, dogs with splenitis, splenic HA and HSA. The results showed that the Ang-2 level in healthy dogs was significantly lower than in the splenitis and splenic HA cases. Moreover, the Ang-2 level was significantly higher in splenic HA than in splenic HSA. Conversely, no significant differences in Ang-2 level were recorded between healthy and splenic HSA dogs, and between splenitis and splenic neoplasms (HA and HSA). No significant correlations were observed between the Ang-2 level and *(i)* the clinical stage, *(ii)* histological growth pattern, and *(iii)* median survival time of splenic HSA dogs. In conclusion, serum Ang-2 concentration is a potentially useful biological marker for the discrimination of dogs with splenitis and splenic HA, as well as for the differentiation of splenic HA from its malignant form, HSA.

KEYWORDS

angiopoietin-2, dog, ELISA, haemangioma, haemangiosarcoma, splenitis

INTRODUCTION

Haemangioma (HA) is among the most common benign splenic neoplasms and haemangiosarcoma (HSA) is the most common malignant endothelial neoplasm of the spleen in dogs (Eberle et al., 2012). Splenic HSA frequently occurs in old-aged and large-breed dogs (Christensen et al., 2009), and more often in males, especially neutered males, than in females (Sabattini and Bettini, 2009). Anatomically, splenic HSA is classified into three stages (Withrow et al., 2013). In Stage 1, the neoplasm is confined to one organ without any rupture, while in Stage 2 the neoplasm has ruptured with or without regional lymph node involvement, but there is no distant metastasis. In Stage 3, the neoplasm has ruptured or invaded other organ structures and there is distant metastasis. However, diagnostic techniques for the determination of splenic HSA are limited. Dogs with splenic HSA are frequently detected at a late stage of the disease, resulting in a short survival time after the initial diagnosis, surgery and chemotherapy, even though chemotherapeutic treatment is able to extend the survival time after splenectomy (Clifford et al., 2000; Goritz et al., 2013). Most dogs with splenic HSA show no specific clinical signs and haematological profiles that are not adequate for the

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specific diagnosis of HSA (Hammer et al., 1991). Additionally, neoplastic mass-to-splenic volume ratio and splenic weight as a percentage of the body weight have also been tested for differentiating between splenic HSA and benign lesions of splenic masses. Both of these ratios had a significantly lower mean in dogs with splenic HSA than in dogs with benign splenic masses (Mallinckrodt and Gottfried, 2011). The advanced imaging procedures, such as dual-phase computed tomography scan, cannot overcome the limitation in the specific diagnosis of splenic HSA and other splenic masses (Jones et al., 2016). The median survival time of splenic HSA-bearing dogs is still less than six months (Sorenmo et al., 2000). Accordingly, the prognosis for dogs with splenic HSA is definitely poor.

In this context, many molecular biological markers have been investigated for use as potential canine splenic HSA markers, such as angiogenic factor related molecules. Immunohistochemistry (IHC) analysis revealed the over-expression of von Willebrand's factor, vimentin, CD31, claudin-5, vascular endothelial growth factor A (VEGF-A) and angiopoietin-2 (Ang-2) in canine splenic HSA, where these molecules play an important role in the neoplastic angiogenesis (Moore et al., 1989; Ferrer et al., 1995; Jakab et al., 2009; Goritz et al., 2013). There was also a study of using plasma cardiac troponin I (cTnI) to differentiate cardiac HSA of dogs from other sites with a significantly higher median plasma cTnI concentration (Chun et al., 2010). Further studies using enzyme-linked immunosorbent assays (ELISA) showed that the concentration of plasma VEGF was significantly elevated in dogs with HSA compared to that in healthy dogs (Clifford et al., 2001), but no differences were found in other splenic abnormalities (Frenz et al., 2014). The serum big endothelin-1 (big ET-1) level was found to be elevated in splenic HSA and significantly downregulated after splenectomy. Moreover, the serum ferritin level increased in all cases of splenic masses (Chikazawa et al., 2013). Even though there were remarkably altered concentrations of big ET-1, ferritin, and VEGF in splenic HSA, the expression of these markers is non-specific and can be altered in other unrelated conditions. For example, the concentration of big ET-1 is also elevated by pulmonic hypertension, renal disease, some neoplasms and inflammatory response (Fukumoto et al., 2015). Therefore, other biological markers are still required to be investigated as potential diagnostic markers for splenic HSA.

Typically, Ang-2 is expressed in the endothelial cells and is released during inflammatory responses and the initiation of angiogenesis (Fiedler et al., 2004; Niedźwiecki et al., 2006). It is known that Ang-2 participates in neoplastic angiogenesis, and it has been shown to be a helpful marker in the diagnosis of neoplastic endothelial cells by IHC analysis (Goritz et al., 2013). Previous studies have shown that Ang-2 might be an initial neoplastic marker for splenic HSA (Goritz et al., 2013). However, recent studies have reported that, compared to healthy dogs, significantly increased Ang-2 plasma levels occur in septicaemic dogs (König et al., 2018, 2019). These findings underlined the fact that Ang-2 level could not be considered as a totally specific neoplastic marker of splenic HA and HSA. The aim of this study was to investigate the serum Ang-2 concentration in healthy dogs, dogs with various benign and malignant splenic neoplasms, and dogs with other, non-neoplastic splenic lesions, and then to extrapolate the relatedness of serum Ang-2 concentrations in dogs with splenitis, splenic HA and HSA.

MATERIALS AND METHODS

Animals

The dogs were separated into three groups. The control group ($n = 9$) consisted of healthy female dogs. The splenic mass group ($n = 40$) was further categorised into the splenic HSA ($n = 13$) and non-HSA ($n = 27$) groups. The essential indication, physical examination, haematological profile, diagnostic imaging profile and median survival time after splenectomy were obtained from the medical records of the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University, Thailand. The occurrence of haemoabdomen and splenic rupture was also recorded during the surgical intervention. All owners were informed and signed consent forms according to the regulation of Chulalongkorn University Animal Care and Use Committee (No. 11310088).

Sample collection

Serum samples. Blood samples were taken from the cephalic vein of healthy dogs and dogs with a splenic mass prior to ovariohysterectomy and splenectomy, respectively. Whole blood samples were collected in a plain tube without anticoagulant and allowed to clot at room temperature for 30 min. The samples were then centrifuged at approximately 1,000 × *g* for 15 min, and the serum was harvested and stored in aliquots at –80 °C until analysis.

Tissue samples. Splenic tissue samples were collected immediately after splenectomy performed at the Surgery Unit of the Small Animal Teaching Hospital and submitted to the Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, for routine histopathological diagnosis. Formalin-fixed paraffin-embedded tissues were sectioned at 4-μm thickness and stained with haematoxylin and eosin.

Pathology and histology

Macroscopic and microscopic findings were investigated. The general appearance, location and size of the neoplasm on the spleen were recorded. Evidence of metastasis was observed intraoperatively by observing the relevant lymph nodes or histologically by presenting the neoplastic intravascular emboli in the blood or lymphatic vessels. When canine splenic HSA was diagnosed, samples were classified according to the histological growth pattern, following the criteria reported previously (Hendrick et al., 1998; Kim et al.,

2015), in: *(i)* capillary, *(ii)* cavernous, *(iii)* solid, or *(iv)* mixed growth pattern. Histopathological investigations were performed by a Thai-board certified veterinary pathologist (ST).

ELISA

A commercial ELISA Kit (Novateinbio; Novatein Biosciences, Woburn, MA, USA; catalogue number BG-CAN10138), specific for canine Ang-2, was used to measure the serum Ang-2 concentration of the dogs. Briefly, all reagents and samples were allowed to warm at room temperature for 30 min. After setting standard solution, sample, and blank wells, the reagents were added following the recommendations from the manufacturer's protocols (https://www.kyvobio.com/-186/dog-canine-angiopoietin-2-ang2-893003357.html). When the colour in the wells developed from blue to yellow, the optical density of the samples was read using an ELISA microplate reader (BioTek EL808, BioTek Instruments, Inc.) at a wavelength of 450 nm. The serum Ang-2 concentrations were determined by comparison with the generated standard curve. All serum samples were measured in duplicate and averaged.

Statistical analyses

The data were evaluated using the SPSS version 22.0 software (IBM Corp, New York, USA). To determine the normal distribution for small sample sizes ($n < 50$), the Shapiro–Wilk test was selected. The serum Ang-2 concentration was compared between healthy dogs and dogs harbouring a splenic mass using the Mann–Whitney U test. The Kruskal–Wallis test was used for comparison among the healthy, splenitis, HA and HSA groups. The correlation between serum Ang-2 concentration and *(i)* the clinical stage, *(ii)* HSA histological growth pattern, and *(iii)* median survival time of splenic HSA dogs, as well as the correlation between survival time and *(i)* HSA clinical stage and *(ii)* HSA growth pattern were analysed by Pearson's linear correlation. A P value <0.05 was considered significant in all cases. Survival times, recorded by telephonic interview at 9 months after splenectomy, were analysed by the Kaplan–Meier method.

RESULTS

Healthy dogs

The average age of the healthy dogs ($n = 9$) was 10.2 months (range 8–12 months, SD = 1.475). This group included different breeds such as Pomeranian, Chihuahua, Shih Tzu, Golden retriever, as well as mongrels. All of these dogs had normal haematology, liver and kidney function test results (Table 4).

Dogs with a splenic mass

The average age of the 40 dogs with splenic masses was 10.9 years (range 4–16 years, SD = 2.92), and consisted of intact males (30%, $n = 12$), castrated males (37%, $n = 15$), intact females (15%, $n = 6$), and spayed females (18%, $n = 7$). The male to female ratio was 2:1. One-third of the dogs belonged to large breeds (32.5%) including Golden retriever ($n = 6$), Labrador retriever ($n = 5$), Shetland sheepdog ($n = 1$) and Siberian husky ($n = 1$). The others were medium- and small-sized breed dogs, comprising mixed breed ($n = 9$), Cocker spaniel ($n = 3$), Shih Tzu ($n = 3$), Beagle ($n = 2$), Poodle ($n = 2$), Bangkaew ($n = 2$), Yorkshire terrier ($n = 1$), Dachshund ($n = 1$), Terrier ($n = 1$), Schnauzer ($n = 1$), French bulldog ($n = 1$), and Bulldog ($n = 1$).

In the splenic HSA group ($n = 13$), the average age was 11.5 years (range 7–14 years, SD = 1.81), with prevalence of males over females (male to female ratio = 2.3:1). Advanced clinical stage 3 was the most frequently found (77%, $n = 10$), followed by clinical stage 2 (15%, $n = 2$), and by clinical stage 1 (8%, $n = 1$). Large breeds (Golden and Labrador retrievers) included the majority of dogs from the HSA group (53.8%, $n = 7$). There was a correlation between HSA clinical stage and survival time ($r = -0.61$, $P = 0.03$). Three splenic HSA-bearing dogs had other concurrent metastatic neoplasms including melanoma, Sertoli cell tumour and adenocarcinoma (Tables 1 and 5).

In the splenic HA group ($n = 6$), the average age was 9.3 years (range 7–13 years, SD = 2.16), with prevalence of males over females (male to female ratio = 2:1). The majority of dogs in this group were medium breeds (Cocker spaniel) (33.3%, $n = 2$) (Table 5).

In the splenitis group ($n = 5$), the average age was 9 years (range 4–16 years, SD = 4.64), with prevalence of males over females (male to female ratio = 1.5:1). Medium breeds (Thai) were more prevalent in this group (20%, $n = 2$) (Table 5).

The dogs in each group showed abnormal haematology findings including anaemia (7/8 in HSA-, 3/6 in HA- and 2/3 in splenitis-bearing dogs), leukocytosis (3/8 in HSA-, 3/6 in HA- and 2/3 in splenitis-bearing dogs), thrombocytopenia (5/8 in HSA-, 3/6 in HA- and 2/3 in splenitis-bearing dogs), and an abnormal coagulopathy demonstrating a high level of D-dimer concentration in HSA dogs (5/5) and a prolonged thrombin time in HA dogs (4/5). *Ehrlichia canis* was detected in one dog with splenitis (Table 4).

Macroscopic findings of splenic masses

The splenic masses appeared as single or multiple nodules of various sizes ranging from 1 × 1 cm to 50 × 70 cm, and located at the head, body or tail of the spleen (Table 5). Most splenic masses were dark red in colour except for some masses diagnosed as lymphoma (case no. 21), malignant fibrous histiocytic sarcoma (case no. 29), plasma cell tumour (case no. 30), or histiocytoma (case no. 31), which presented with a white colour. Fourteen masses (35%) were ruptured with the highest incidence in splenic HSA (22.5%, $n = 9$), then in histiocytic splenitis ($n = 2$), lymphoma ($n = 1$), mast cell tumour ($n = 1$) and plasma cell tumour ($n = 1$). Metastasis was macroscopically detected in HSA-bearing dogs ($n = 9$).

Table 1. Signalment, clinical staging, evidence of neoplasm rupture, survival time and Ang-2 level of dogs with splenic haemangiosarcoma (HSA)

Dog number[a]	Breed	Age (years)	Sex[b]	Clinical staging[c]	Neoplasm rupture	Survival time[d] (days)	Ang-2 level (ng/mL)
1	Labrador retriever	10	Mc	3	Yes	56	1.50
2	Golden retriever	7	Mc	3	Yes	51	2.50
3	Labrador retriever	12	Mc	3	No	57	6.00
4	Beagle	12	M	3	Yes	199	1.25
5	Golden retriever	9	M	3	Yes	0	1.10
6	Yorkshire terrier	12	Mc	1	No	N/A	1.40
7	Beagle	12	Mc	2	No	90	7.70
8	Labrador retriever	12	Mc	2	No	264	3.00
9	Mixed	12	F	3	Yes	6	1.56
10	Labrador retriever	12	F	3	Yes	5	1.10
11	Mixed	14	F	3	Yes	6	2.00
12	Labrador retriever	12	M	3	Yes	73	1.40
13	Mixed	13	F	3	Yes	9	0.60

[a] Dog no. 3 had splenic HSA and metastatic melanoma; Dog no. 7 had splenic HSA and metastatic Sertoli cell tumour; Dog no. 13 had splenic HSA and metastatic adenocarcinoma.
[b] M, male; Mc, castrated male; F, female.
[c] Stage 1: tumour confined to one organ without rupture; Stage 2: tumour rupture with or without regional lymph node involvement and no evidence of distant metastasis; Stage 3, ruptured tumour or tumour invading another structure with distant metastasis.
[d] Survival time was recorded by telephonic interview at 9 months after splenectomy; N/A, data not available.

Histopathology of splenic masses

The biopsy results from 40 splenic masses were categorised into three groups: non-neoplastic (30%, $n = 12$), benign neoplasm (17.5%, $n = 7$), and malignant neoplasm (52.5%, $n = 21$) (Table 2). Splenitis ($n = 5$), HA ($n = 6$) and HSA ($n = 13$), respectively, were the most frequently found lesions in each group. Among the histological growth patterns of the 13 HSA dogs, the cavernous type was the most common (46.1%; $n = 6$), followed by the solid type (23.1%; $n = 3$), while the capillary and the mixed types were equally the least commonly found (15.4%; $n = 2$) (Table 3).

Table 2. Histopathological results of 40 splenic masses

Diagnosis	Number (%)
Non-tumour	12 (30%)
Splenitis	5 (12.5%)
Splenic nodular hyperplasia	3 (7.5%)
Haematoma	2 (5%)
Splenic congestion	1 (2.5%)
Splenic infarction	1 (2.5%)
Benign tumour	7 (17.5%)
Haemangioma	6 (15%)
Histiocytoma	1 (2.5%)
Malignant tumour	21 (52.5%)
Haemangiosarcoma	13 (32.5%)
Lymphoma	4 (10%)
Plasma cell tumour	1 (2.5%)
Mast cell tumour	1 (2.5%)
Granulosa cell tumour	1 (2.5%)
Malignant fibrous histiocytic sarcoma	1 (2.5%)

Serum Ang-2 concentration

The median serum Ang-2 concentration of healthy dogs (1.05 ng/mL; range 0.50–3.25 ng/mL, Table 5) was significantly lower than in dogs with a splenic mass, regardless of the specific lesion type (1.78 ng/mL; range 0.60–11.80 ng/mL; $P = 0.03$) (Fig. 1a). With respect to the neoplasms of endothelial origin, the median serum Ang-2 concentration in dogs with splenic HA (4.85 ng/mL; range 1.45–11.80 ng/mL) was higher than in those with HSA (1.45 ng/mL; range 1.10–3.00 ng/mL; $P = 0.02$) or in healthy dogs ($P = 0.03$). Moreover, the median serum Ang-2 concentration in dogs with splenitis (4.5 ng/mL; range 1.10–9.00 ng/mL) was also significantly higher than in healthy dogs ($P = 0.01$). However, the serum Ang-2 level was not significantly different

Table 3. Growth pattern and survival time of 13 dogs with haemangiosarcoma

Growth pattern	Number (%)	Survival time (days)		
		Mean ± standard deviation	Range	Median
Cavernous	6 (46.2%)	55 ± 22.10	5–90	9
Solid	3 (23.1%)	102 ± 48.52	51–199	56
Capillary	2 (15.4%)	135 ± 129	6–264	6
Mixed	2 (15.4%)	28 ± 28.50	0–57	0

Table 4. Haematological and clinical chemistry profiles of 9 healthy dogs and 40 dogs with splenic masses

			Parameters[b]														
No.[a]	Breed	Histopathological diagnosis	RBC (10^6/μL) [5.5–8.5]	Hct (%) [37–55]	WBC (10^3/μL) [6–17]	Plt (10^3/μL) [200–500]	BP	PT (Sec) [9–14]	PTT (Sec) [17–30]	TT (Sec) [4–10]	D-dimer (ng/mL) [200–500]	BUN (mg/dL) [7–25]	Cr (mg/dL) [0.3–1.4]	ALT (U/L) [10–118]	ALP (U/L) [20–150]	TP (g/dL) [5.4–8.2]	Alb (g/dL) [2.5–4.4]
Healthy group																	
1	Bichon frise	–	7.42	43.2	14.5	365	Negative	N/D	N/D	N/D	N/D	13	0.8	63	46	6.7	3.1
2	Pomeranian	–	5.53	33.2	10.2	417	N/D	N/D	N/D	N/D	N/D	N/D	0.7	27	N/D	5.7	2.8
3	Pomeranian	–	6.63	42.5	8.3	486	N/D	N/D	N/D	N/D	N/D	N/D	0.7	23	N/D	6.5	3.1
4	Pug	–	7.22	47.2	11.4	368	Negative	N/D	N/D	N/D	N/D	18	0.6	26	61	7.2	2.9
5	Pit bull	–	6.99	42.5	7.8	182	N/D	N/D	N/D	N/D	N/D	N/D	1	63	N/D	6.6	N/D
6	Mixed	–	5.19	39.5	8.4	389	N/D	N/D	N/D	N/D	N/D	17	1	93	140	7.5	3.4
7	Siberian husky	–	6.47	42.4	12	172	N/D	N/D	N/D	N/D	N/D	N/D	1.1	35	N/D	7	3
8	Labrador retriever	–	6.1	41	16	203	N/D	N/D	N/D	N/D	N/D	20	0.7	26	132	6.5	2.9
9	Golden retriever	–	5.7	39	11.2	243	N/D	N/D	N/D	N/D	N/D	20	1	35	96	7	3.2
Splenic mass group																	
1	Labrador retriever	Haemangiosarcoma	3.96	25.4	25.6	154	Negative	<7	<15	N/D	985	17	0.8	26	58	5.6	2.4
2	Golden retriever	Haemangiosarcoma	5.64	34.9	18.5	163	Negative	<7	<15	N/D	52,939	14	1.1	34	99	6.3	1.9
3	Labrador retriever	Haemangiosarcoma and metastatic melanoma	5.08	36.9	21.4	284	Negative	<7	<15	N/D	2,634	20	1.4	34	130	7.1	2.7
4	Beagle	Haemangiosarcoma	4.62	30.9	15.5	111	Negative	<7	<15	N/D	10,830	9	0.5	42	76	4.7	2.7
5	Golden retriever	Haemangiosarcoma	1.8	14.3	25.9	45	Negative	26.2	25.9	N/D	30,968	27	2.2	73	107	6.5	2.6
6	Yorkshire terrier	Haemangiosarcoma	4.98	36.2	13.8	283	Negative	N/D	N/D	N/D	N/D	15.8	0.5	276	125	6	3.2
7	Beagle	Haemangiosarcoma and metastatic Sertoli cell tumour															
8	Labrador retriever	Haemangiosarcoma	5.1	37	14.7	364	Negative	N/D	N/D	N/D	N/D	8	0.6	88	370	4.2	1.8
9	Mixed	Haemangiosarcoma															
10	Labrador retriever	Haemangiosarcoma															
11	Mixed	Haemangiosarcoma															
12	Labrador retriever	Haemangiosarcoma	2.1	15.3	16.5	116	Negative	7	21	N/D	N/D	25	2.2	22	96	7.3	2.4

(continued)

Table 4. Continued

No.[a]	Breed	Histopathological diagnosis	Parameters[b] RBC (10^6/μL) [5.5–8.5]	Hct (%) [37–55]	WBC (10^3/μL) [6–17]	Plt (10^3/μL) [200–500]	BP	PT (Sec) [9–14]	PTT (Sec) [17–30]	TT (Sec) [4–10]	D-dimer (ng/mL) [200–500]	BUN (mg/dL) [7–25]	Cr (mg/dL) [0.3–1.4]	ALT (U/L) [10–118]	ALP (U/L) [20–150]	TP (g/dL) [5.4–8.2]	Alb (g/dL) [2.5–4.4]
13	Mixed	Haemangiosarcoma and metastatic adenocarcinoma															
14	Cocker spaniel	Haemangioma	2.6	20	25.8	229	Negative	8	26	12	N/D	14	0.6	25	45	6.7	2.4
15	Dachshund	Haemangioma	6.7	46	94.2	363	Negative	6	16	12	N/D	12	0.7	131	701	7.5	4.1
16	Golden retriever	Haemangioma	6.3	37	5.56	202	Negative	<7	<15	23.5	N/D	13.1	0.9	26	37	7.2	3.1
17	Cocker spaniel	Haemangioma	4.6	22.7	52.5	182	Negative	6	14	10	N/D	11	0.7	12.4	233	6.3	2
18	Siberian husky	Haemangioma	4.1	24	6.13	90	Negative	<7	<15	20	336	15.9	1.5	22	75	5.2	1.8
19	Bulldog	Haemangioma	6.48	42.9	7.8	195	Negative	N/D	N/D	N/D	1,303	10	1.6	86	194	8.9	2.7
20	Golden retriever	Lymphoma	5	34.4	11.1	511	N/D	N/D	N/D	N/D	N/D	17	1.1	100	350	6.6	2.8
21	Golden retriever	Lymphoma															
22	Cocker spaniel	Lymphoma															
23	Shih Tzu	Lymphoma															
24	Poodle	Haematoma	7.2	49	5.72	270	Negative	8	14	8	N/D	14	0.8	75	57	4.8	2.4
25	Terria	Haematoma	5.64	43	11.5	273	Negative	7	16	15	N/D	44	1.5	127	859	5.8	2.6
26	Shih Tzu	Nodular hyperplasia	6.52	46.9	15.5	314	N/D	N/D	N/D	N/D	N/D	12	0.8	70	105	6.8	2.5
27	Mixed	Nodular hyperplasia	2.19	17.4	36.3	162	Negative	7.2	11.1	N/D	N/D	21	0.7	12	93	4.8	2.4
28	Shih Tzu	Nodular hyperplasia	5.88	36.5	6	498	N/D	N/D	N/D	N/D	N/D	30	0.8	71	103	6.3	2.6
29	Shetland sheepdog	Malignant fibrous histiocytic sarcoma	5.9	41	6.73	470	Negative	<7	<15	18	N/D	9	0.6	51	441	7	2.8
30	Mixed	Plasma cell tumour	4.6	32	6.73	30	*E. canis*	N/D	N/D	N/D	N/D	42	1.2	46	70	11.9	1.5
31	Mixed	Histiocytoma	5.3	31	10.9	209	Negative	N/D	N/D	N/D	N/D	12	1	23	50	9.6	2.1
32	Schnauzer	Splenic infarction	6.48	42.6	13.3	279	Negative	<7	<15	17	1,493	12.7	0.9	40	124	5.6	2.5
33	French bulldog	Splenic congestion	6	41.5	26	143	Negative	N/D	N/D	N/D	N/D	6	1.4	31	82	6	N/D
34	Mixed	Metastatic mast cell tumour	8.65	47.3	11.8	165	Negative	N/D	N/D	N/D	N/D	26	1.1	15	197	7	2.7
35	Mixed	Metastatic granulosa cell tumour															
36	Golden retriever	Plasmacytic splenitis															

(continued)

Table 4. Continued

No.[a]	Breed	Histopathological diagnosis	Parameters[b]													
			RBC (10^6/µL) [5.5–8.5]	Hct (%) [37–55]	WBC (10^3/µL) [6–17]	Plt (10^3/µL) [200–500]	BP	PT (Sec) [9–14]	PTT (Sec) [17–30]	TT (Sec) [4–10]	D-dimer (ng/mL) [200–500]	BUN (mg/dL) [7–25]	Cr (mg/dL) [0.3–1.4]	ALT (U/L) [10–118]	ALP (U/L) [20–150]	TP (g/dL) [5.4–8.2]	Alb (g/dL) [2.5–4.4]
37	Mixed	Histiocytic splenitis															
38	Thai	Histiocytic splenitis	2.3	19.5	10.2	47	*E. canis*	N/D	N/D	N/D	N/D	45.2	0.9	55	128	7.2	2.3
39	Poodle	Suppurative splenitis	4.5	30	73.6	33	Negative	N/D	N/D	N/D	N/D	13	0.5	25	895	6	1.8
40	Thai	Necrotic splenitis	7	39	22.8	248	Negative	5	16	13	N/D	14	0.8	53	68	7.1	2.3

[a] Data could not retrieved from dogs no. 7, 9, 10, 11, 13, 21, 22, 23, 35, 36 and 37 from the splenic mass group.

[b] *Abbreviations:* RBC, Red blood cells; Hct, Haematocrit; WBC, White blood cells; Plt, Platelets; BP, Blood parasite; PT, Prothrombin time; PTT, Partial thromboplastin time; TT, Thrombin time; BUN, Blood urea nitrogen; Cr, Creatinine; ALT, Alanine aminotransferase; ALP, Alkaline phosphatase; TP, Total protein; Alb, Albumin; N/D, not determined; *E. canis*, *Ehrlichia canis*.

between splenic HSA and healthy dogs ($P = 0.20$), and dogs with splenitis and HA ($P = 0.86$) and HSA ($P = 0.11$) (Fig. 1b).

There was no correlation between the serum Ang-2 level in dogs with splenic HSA and the clinical stage of HSA ($R = -0.25$, $P = 0.42$), HSA histological growth pattern ($r = 0.27$, $P = 0.38$), and median survival time of splenic HSA dogs ($r = -0.20$, $P = 0.53$). In addition, elevated serum Ang-2 levels were observed in HSA dogs with metastasis from melanoma (6.00 ng/mL) and Sertoli cell tumour (7.70 ng/mL), but not from adenocarcinoma (0.60 ng/mL) (Table 5).

Survival time of dogs with splenic HSA

The survival time of the affected dogs varied. Half of them remained apparently healthy for more than 50 days (53.8%, $n = 7$), whereas five of the others succumbed after less than 10 days (there were no survival data for one dog). The survival time after splenectomy was recorded in 12 of the splenic HSA dogs (Table 1). The mean survival time was 80.6 days (SD = 25.9), while the median survival time was 56 days (range 0–264 days) (Fig. 2). HSA dogs with a capillary growth pattern seemed to have a longer survival time, followed by those with a solid, cavernous, and mixed growth type lesion, in decreasing order. However, the survival time was independent of the clinical stage ($r = -0.61$, $P = 0.03$) and the observed growth pattern of HSA ($r = -0.13$, $P = 0.69$, Table 3). The survival time of dogs with a ruptured HSA was shorter than that of dogs with a non-ruptured neoplasm ($r = 0.59$, $P = 0.04$).

DISCUSSION

In dogs, Ang-2 has been shown to be an informative biological marker for systemic inflammatory response syndrome (SIRS), sepsis, and neoplastic endothelial cells (Kato et al., 2006; Goritz et al., 2013; König et al., 2018, 2019). Thus, it was of interest to determine if Ang-2 might also be a useful serum marker for canine splenitis, splenic HA and HSA. In this study, the serum Ang-2 concentration was investigated using a commercial ELISA kit specific for canine Ang-2 in both healthy dogs and dogs with various splenic abnormalities. The dogs with splenitis exhibited a higher serum Ang-2 concentration compared to the healthy group. This observation is in accordance with previous reports that have indicated a significantly higher Ang-2 concentration in the blood of dogs with SIRS and sepsis than in that of healthy dogs (König et al., 2018, 2019). In addition, dogs with splenic HSA showed a low serum Ang-2 concentration compared to the HA group in our study. This might have been caused by the intralesional necrosis of the splenic HSA neoplasm followed by endothelial cell destruction and loss. It has been hypothesised that although Ang-2 plays a crucial role in angiogenesis during neoplastic growth via the angioprotein-TIE signalling, its expression may be decreased in neoplasms of

Table 5. Biological data and serum angiopoietin-2 (Ang-2) levels of 9 healthy dogs and 40 dogs with splenic masses

No.	Breed	Age (y)	Sex[a]	Location of mass at the spleen	Gross lesions	Size (cm)	Neoplastic rupture	Histopathological diagnosis	Metastasis	Ang-2 level (ng/mL)
Healthy group										
1	Bichon frise	1	F	–	–	–	–	–	–	0.90
2	Pomeranian	1	F	–	–	–	–	–	–	0.70
3	Pomeranian	1	F	–	–	–	–	–	–	3.00
4	Pug	3	F	–	–	–	–	–	–	1.00
5	Pit bull	1	F	–	–	–	–	–	–	1.05
6	Mixed	8	F	–	–	–	–	–	–	2.75
7	Siberian husky	1	F	–	–	–	–	–	–	1.10
8	Labrador retriever	3	F	–	–	–	–	–	–	0.50
9	Golden retriever	4	F	–	–	–	–	–	–	3.25
Splenic mass group										
1	Labrador retriever	10	Mc	Body to tail	Single nodule	50 × 60	Yes	Haemangiosarcoma	Yes	1.50
2	Golden retriever	7	Mc	Body	Single nodule	10 × 15	Yes	Haemangiosarcoma	Yes	2.50
3	Labrador retriever	12	Mc	Head	Multiple nodules	10 × 15	No	Haemangiosarcoma and metastatic melanoma	No	6.00
4	Beagle	12	M	Body	Multiple nodules	10 × 12	Yes	Haemangiosarcoma	Yes	1.25
5	Golden retriever	9	M	Body to tail	Large multiple masses	50 × 70	Yes	Haemangiosarcoma	Yes	1.10
6	Yorkshire terrier	12	Mc	Tail	Single nodule	2 × 2	No	Haemangiosarcoma	No	1.40
7	Beagle	12	Mc	Body	Single nodule	4 × 6	No	Haemangiosarcoma and metastatic Sertoli cell tumour	No	7.70
8	Labrador retriever	12	Mc	Tail	Single nodule	15 × 15	No	Haemangiosarcoma	No	3.00
9	Mixed	12	F	Tail	Single nodule	5 × 6	Yes	Haemangiosarcoma	Yes	1.56
10	Labrador retriever	12	F	Body to tail	Multiple nodules with a large single mass	50 × 60	Yes	Haemangiosarcoma	Yes	1.10
11	Mixed	14	F	Tail	Single nodule	10 × 12	Yes	Haemangiosarcoma	Yes	2.00
12	Labrador retriever	12	M	Body to tail	Multiple nodules with a large single mass	30 × 50	Yes	Haemangiosarcoma	Yes	1.40
13	Mixed	13	F	Head	Single nodule	7 × 8	Yes	Haemangiosarcoma and metastatic adenocarcinoma	Yes	0.60
14	Cocker spaniel	13	Mc	Body	Single nodule	15 × 30	No	Haemangioma	No	2.00
15	Dachshund	8	M	Head	Single nodule	8 × 8	No	Haemangioma	No	11.80

(continued)

Table 5. Continued

No.	Breed	Age (y)	Sex[a]	Location of mass at the spleen	Gross lesions	Size (cm)	Neoplastic rupture	Histopathological diagnosis	Metastasis	Ang-2 level (ng/mL)
16	Golden retriever	8	M	Tail	Single nodule	8 × 8	No	Haemangioma	No	1.45
17	Cocker spaniel	10	Fs	Tail	Single nodule	10 × 10	No	Haemangioma	No	7.50
18	Siberian husky	10	Fs	Tail	Single nodule	3 × 5	No	Haemangioma	No	7.00
19	Bulldog	7	Mc	Body	Single nodule	10 × 10	No	Haemangioma	No	2.70
20	Golden retriever	5	M	Body	Single nodule	3 × 5	No	Lymphoma	No	1.50
21	Golden retriever	8	Mc	Body	Single nodule	20 × 25	No	Lymphoma	No	1.56
22	Cocker spaniel	10	F	Tail	Single nodule	25 × 35	No	Lymphoma	No	3.13
23	Shih Tzu	15	M	Tail	Single nodule	8 × 6	Yes	Lymphoma	No	1.56
24	Poodle	12	Fs	Tail	Single nodule	3 × 3	No	Haematoma	No	1.00
25	Terria	16	M	Tail	Single nodule	7 × 8	No	Haematoma	No	8.50
26	Shih Tzu	11	Mc	Body	Single nodule	1 × 1	No	Nodular hyperplasia	No	2.25
27	Mixed	14	Mc	Diffuse	Multiple nodules	4 × 5	No	Nodular hyperplasia	No	1.05
28	Shih Tzu	6	Mc	Tail	Single nodule	3 × 4	No	Nodular hyperplasia	No	0.95
29	Shetland sheepdog	11	M	Tail	Single nodule	4 × 3	No	Malignant fibrous histiocytic sarcoma	No	1.15
30	Mixed	10	Fs	Diffuse	Large multiple nodules	15 × 20	Yes	Plasma cell tumour	No	2.00
31	Mixed	12	M	Body	Single nodule	20 × 30	No	Histiocytoma	No	1.35
32	Schnauzer	11	Fs	Tail	Single nodule	5 × 5	No	Splenic infarction	No	6.30
33	French bulldog	9	Mc	Body	Single nodule	4 × 5	No	Splenic congestion	No	1.55
34	Mixed	5	F	Diffuse	Multiple nodules	1 × 2	Yes	Metastatic mast cell tumour	Yes	2.25
35	Mixed	8	Fs	Tail	Single nodule	10 × 12	No	Metastatic granulosa cell tumour	Yes	1.40
36	Golden retriever	7	F	Tail	Single nodule	15 × 20	No	Plasmacytic splenitis	No	6.00
37	Mixed	16	M	Tail	Single nodule	6 × 7	Yes	Histiocytic splenitis	No	9.00
38	Thai	7	M	Diffuse	Multiple nodules	15 × 33	Yes	Histiocytic splenitis	No	4.00
39	Poodle	4	M	Tail	Single nodule	2 × 3	No	Suppurative splenitis	No	1.10
40	Thai	11	F	Body	Single nodule	12 × 12	No	Necrotic splenitis	No	4.50

[a] M, male; Mc, castrated male; F, female; Fs, spayed female; y, years.

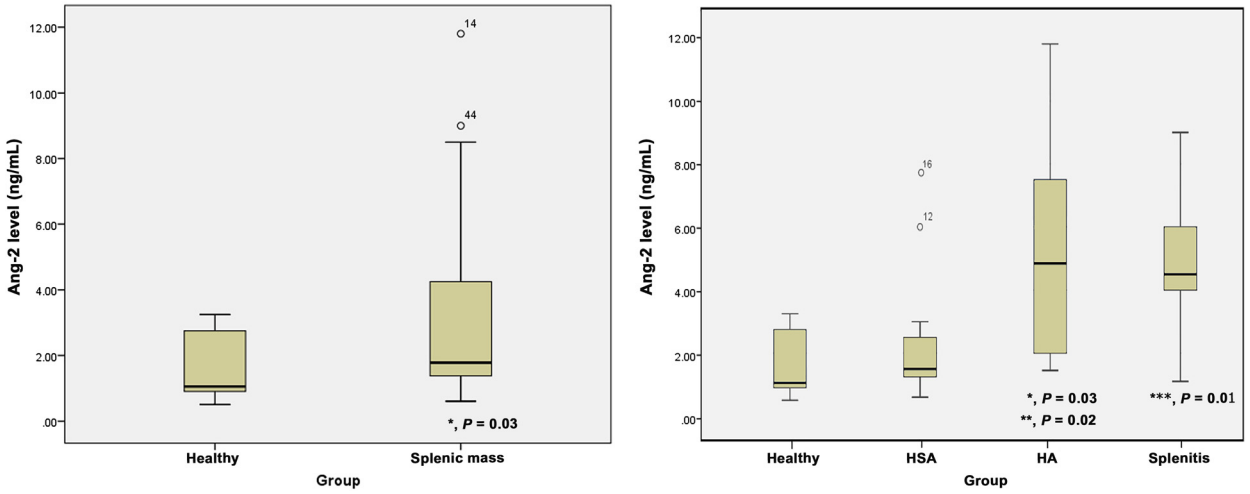

Fig. 1. Box plot showed the serum Ang-2 levels in (a) healthy group and splenic mass-bearing dogs, with a significant difference (*, $P = 0.03$) between the two groups; and in (b) healthy group, splenic haemangiosarcoma (HSA) group, haemangioma (HA) group, and splenitis group, with a significant difference between the healthy and the HA groups (*, $P = 0.03$), between the HSA and the HA groups (**, $P = 0.02$), and between the healthy and the splenitis groups (***, $P = 0.01$). Data are shown as the mean ± 1 SD, derived from double replicates

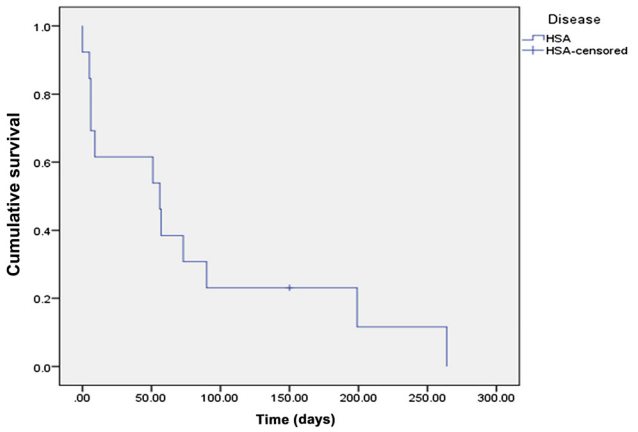

Fig. 2. Kaplan–Meier survival plot of 13 dogs with splenic haemangiosarcoma (HSA). The median survival time was 56 days (mean 80 days, range 0–264 days). The end follow-up time-point was at 9 months after splenectomy

endothelial cell origin as found in a study on human angiosarcoma (Buehler et al., 2013). This hypothesis is in agreement with our finding showing a low Ang-2 level in dogs with splenic HSA.

In contrast, expression of the Ang-2 gene has been reported to be increased in several spontaneous canine neoplasms, such as simple mammary carcinoma, hepatocellular carcinoma, and mastocytoma (Kato et al., 2006; Tanabe et al., 2019). This probably reflects that Ang-2 plays a role in neoplastic angiogenesis, which is driven by neoplastic-associated endothelial cells (Fagiani and Christofori, 2013). Our study found that serum Ang-2 levels tended to be elevated in HSA dogs with metastasis from other neoplasms (melanoma and Sertoli cell tumours), which may be associated with new blood vessel formation in metastatic neoplasms. In addition, in the current study there was no correlation between the serum Ang-2 concentration and all of HSA clinical stage, histological growth pattern, and median survival time. Therefore, the use of serum Ang-2 concentration as a predictive marker of HSA-bearing dogs could not be concluded from this study. However, an elevated Ang-2 concentration has also been noted in another study in ischaemia-induced coronary angiogenesis in dogs (Matsunaga et al., 2003). Therefore, those concurrent conditions should be taken into account during interpretation of the Ang-2 level in dogs harbouring a splenic mass.

The misdiagnosis of splenic HSA to other types of splenic neoplasms can occur by the finding of haematoma, necrosis, and haemorrhage by histopathological examination when insufficient numbers of samples are processed. Therefore, veterinary pathologists are suggested to examine a number of sections in order to improve the diagnostic accuracy. A previous study has suggested that five sections can be considered sufficient for histopathological diagnosis (Herman et al., 2019). However, we examined fewer sections from the submitted samples, and this is an eventual limitation.

In this study, the most common growth pattern of canine splenic HSA was the cavernous type, although it was previously reported that a mixture of cavernous, capillary and solid growth patterns was the most commonly found form in canine splenic HSA (Goritz et al., 2013). However, the sample collection site might affect the microscopic findings and classification of the growth pattern. Therefore, specimens should be collected from several areas of the splenic mass. This should be taken into account in the next study.

The median survival time of dogs with splenic HSA was relatively short after their initial diagnosis or splenectomy, and it was not related to the clinical stage or histological growth pattern of the splenic HSA (Wood et al., 1998), which are in agreement with the findings of our study, whereas haemoperitoneum is probably associated with splenic HSA (Hammond and Pesillo-Crosby, 2008).

However, a ruptured splenic HSA at any clinical stage has a higher probability of a short survival time, which was also noted in this study. Additionally, metastasis of HSA to distant vital organs, such as the heart or liver, leads to a shorter survival time.

This is in accordance with our study that revealed a higher serum Ang-2 concentration in splenic HA dogs than in HSA dogs. Splenic HA may lead to local hypoxia at the splenic mass, and cause nearby endothelial cells to proliferate (Cao et al., 2007). A strong Ang-2 expression level and secretion would occur after endothelial cell activation and would cause vasculature dissociation, endothelial cell apoptosis, and vessel regression. The tissue mass would then become hypoxic and so leads to the upregulation of angiogenic factors, including Ang-2 (Fagiani and Christofori, 2013). Moreover, in this study dogs with splenitis also showed an elevated Ang-2 level, which may have resulted from the increased Ang-2 expression during inflammation because Ang-2 is important for the inflammatory remodelling of blood vessels (Fiedler et al., 2004). Additionally, a previous study has also reported an increased plasma Ang-2 concentration in septicaemic dogs and dogs with SIRS (König et al., 2018, 2019).

In conclusion, our study showed that measuring the serum Ang-2 level is potentially useful for the diagnosis of canine splenic HA, but not for HSA, nor for splenitis. A larger sample size will be required for a further study of the complementary diagnostic value of Ang-2 level in both benign and malignant splenic neoplasms.

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