การศึกษาเปรียบเทียบระดับความเข้มข้นของแองจิโอพอยอิตินทูในเลือดสุนัขปกติและสุนัขที่เป็นมะเร็งเนื้อเยื่อผนังหลอดเลือดที่ม้าม

นางสาวศุภิสรา วงศ์สุทธาวาส

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

บทคัดย่อและแฟ้มข้อมูลฉบับเต็มของวิทยานิพนธ์ตั้งแต่ปีการศึกษา 2554 ที่ให้บริการในคลังปัญญาจุฬาฯ (CUIR)
เป็นแฟ้มข้อมูลของนิสิตเจ้าของวิทยานิพนธ์ ที่ส่งผ่านทางบัณฑิตวิทยาลัย
The abstract and full text of theses from the academic year 2011 in Chulalongkorn University Intellectual Repository (CUIR)
are the thesis authors' files submitted through the University Graduate School.

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาศัลยศาสตร์ทางสัตวแพทย์ ภาควิชาศัลยศาสตร์
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2558
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

COMPARATIVE STUDY OF SERUM ANGIOPOIETIN - 2 CONCENTRATION IN HEALTHY AND SPLENIC HEMANGIOSARCOMA DOGS

Miss Supissara Wongsuttawas

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Veterinary Surgery
Department of Veterinary Surgery
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2015

Copyright of Chulalongkorn University

Thesis Title	COMPARATIVE STUDY OF SERUM ANGIOPOIETIN - 2 CONCENTRATION IN HEALTHY AND SPLENIC HEMANGIOSARCOMA DOGS
By	Miss Supissara Wongsuttawas
Field of Study	Veterinary Surgery
Thesis Advisor	Assistant Professor Sumit Durongphongtorn, D.V.M., D.V.Sc., D.T.B.V.S.
Thesis Co-Advisor	Associate Professor Somporn Techangamsuwan, D.V.M., Ph.D., D.T.B.V.P.

Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Veterinary Science
(Professor Roongroje Thanawongnuwech, D.V.M., Ph.D., D.T.B.V.P.)

THESIS COMMITTEE

.....Chairman
(Professor Marissak Kalpravidh, B.Sc., D.V.M., M.S., Ph.D., D.T.B.V.S.)

.....Thesis Advisor
(Assistant Professor Sumit Durongphongtorn, D.V.M., D.V.Sc., D.T.B.V.S.)

.....Thesis Co-Advisor
(Associate Professor Somporn Techangamsuwan, D.V.M., Ph.D., D.T.B.V.P.)

.....Examiner
(Associate Professor Nopadon Pirarat, D.V.M., Ph.D., D.T.B.V.P.)

.....External Examiner
(Associate Professor Preenun Jitasombuti, D.V.M., M.S., D.T.B.V.S.)

ศุภิสรา วงศ์สุทธาวาส : การศึกษาเปรียบเทียบระดับความเข้มข้นของแองจิโอพอยอิตินทูในเลือดสุนัขปกติและสุนัขที่เป็นมะเร็งเนื้อเยื่อผนังหลอดเลือดที่ม้าม (COMPARATIVE STUDY OF SERUM ANGIOPOIETIN - 2 CONCENTRATION IN HEALTHY AND SPLENIC HEMANGIOSARCOMA DOGS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. น.สพ. สุมิตร ดุรงค์พงษ์ธร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. สพ.ญ. ดร. สมพร เตชะงามสุวรรณ, 81 หน้า.

มะเร็งเนื้อเยื่อผนังหลอดเลือดที่ม้ามเป็นเนื้องอกชนิดร้ายแรงที่พบได้บ่อยในสุนัข โดยพบมะเร็งชนิดนี้หนึ่งในสามของเนื้องอกในม้าม สุนัขจะแสดงอาการที่ไม่จำเพาะเจาะจงต่อโรค ทำให้มีข้อจำกัดในการวินิจฉัย ดังนั้นคณะผู้วิจัยเล็งเห็นว่า หากสามารถตรวจพบมะเร็งเนื้อเยื่อผนังหลอดเลือดที่ม้ามได้ในระยะแรกย่อมสามารถเพิ่มประสิทธิภาพการรักษามะเร็งชนิดนี้ได้ จึงมีความสนใจศึกษาการแสดงออกของโปรตีนที่อาจจำเพาะต่อมะเร็งเนื้อเยื่อผนังหลอดเลือดที่ม้าม ซึ่งการศึกษาในกลุ่มเหล่านี้ยังมีไม่มากนัก งานวิจัยนี้จึงศึกษาระดับความเข้มข้นของแองจิโอพอยอิตินทูในเลือดของสุนัขที่เป็นมะเร็งเนื้อเยื่อผนังหลอดเลือดที่ม้าม โดยทำการเก็บเลือดจากสุนัขที่มีความผิดปกติของม้ามจำนวน 40 ตัว ทั้งก่อนและหลังผ่าตัดม้าม 10 วัน มีกลุ่มควบคุมคือกลุ่มสุนัขเพศเมียสุขภาพแข็งแรง 10 ตัวทั้งก่อนและหลังผ่าตัดทำหมันปกติ 10 วัน จากนั้นนำเลือดไปปั่นแยกซีรั่ม และวิเคราะห์หาระดับความเข้มข้นของแองจิโอพอยอิตินทูโดยวิธีอีไลซ่า โดยเทียบกับความเข้มข้นอ้างอิงจากกราฟมาตรฐาน นอกจากนี้ในส่วนของชิ้นเนื้อม้ามได้นำไปอ่านผลแยกแยะชนิดของความผิดปกติทางจุลพยาธิวิทยา โดยในกลุ่มมะเร็งเนื้อเยื่อผนังหลอดเลือดที่ม้ามจะมีการจำแนกลักษณะรูปแบบการเจริญเติบโตของเนื้อเยื่อ ร่วมกับลักษณะอื่นๆ ที่บ่งบอกถึงความผิดปกติของเนื้อเยื่อม้าม ผลการวิจัยพบว่าระดับความเข้มข้นของแองจิโอพอยอิตินทูไม่มีแตกต่างอย่างมีนัยสำคัญทางสถิติ (1) ระหว่างสุนัขกลุ่มควบคุมและสุนัขกลุ่มที่มีความผิดปกติที่ม้ามทั้งก่อนและหลังผ่าตัด 10 วัน (2) ระหว่างกลุ่มที่ม้ามไม่เป็นเนื้องอกและเป็นเนื้องอก (3) ระหว่างกลุ่มที่ม้ามเป็นเนื้องอกธรรมดาและมะเร็งร้ายแรง (4) ระหว่างสุนัขกลุ่มควบคุมและสุนัขกลุ่มที่เป็นมะเร็งเนื้อเยื่อผนังหลอดเลือดที่ม้าม (5) ระหว่างสุนัขกลุ่มที่เป็นมะเร็งเนื้อเยื่อผนังหลอดเลือดที่ม้ามที่ระยะต่างๆ ของโรค ($P>0.05$) แต่พบว่าเมื่อเทียบระดับความเข้มข้นของแองจิโอพอยอิตินทูในสุนัขที่เป็นมะเร็งเนื้อเยื่อผนังหลอดเลือดที่ม้ามแบบไม่ร้ายแรงกับสุนัขกลุ่มควบคุมและสุนัขกลุ่มที่เป็นมะเร็งเนื้อเยื่อผนังหลอดเลือดที่ม้ามแบบร้ายแรงกลับพบว่ามีการเพิ่มขึ้นอย่างมีนัยสาคัญทางสถิติ ($P<0.05$) จึงอาจสรุปได้ว่าปัจจัยในการสร้างหลอดเลือดชนิดแองจิโอพอยอิตินทูไม่มีความจำเพาะต่อมะเร็งเนื้อเยื่อผนังหลอดเลือดที่ม้าม

ภาควิชา ศัลยศาสตร์ ลายมือชื่อนิสิต .....

สาขาวิชา ศัลยศาสตร์ทางสัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5675324731 : MAJOR VETERINARY SURGERY
KEYWORDS: ปัจจัยในการสร้างหลอดเลือด แองจิโอโพอิตินทู มะเร็งเนื้อเยื่อผนังหลอดเลือดที่ม้าม อีไลซา / ANGIOGENIC FACTORS, ANGIOPOIETIN-2, CANINE SPLENIC HEMANGIOSARCOMA, ELISA

SUPISSARA WONGSUTTAWAS: COMPARATIVE STUDY OF SERUM ANGIOPOIETIN - 2 CONCENTRATION IN HEALTHY AND SPLENIC HEMANGIOSARCOMA DOGS. ADVISOR: ASST. PROF. SUMIT DURONGPHONGTORN, D.V.M., D.V.Sc., D.T.B.V.S., CO-ADVISOR: ASSOC. PROF. SOMPORN TECHANGAMSUWAN, D.V.M., Ph.D., D.T.B.V.P., 81 pp.

Hemangiosarcoma (HSA) is a common splenic malignant tumor in dogs. HSA accounts for approximately one third of dogs with splenic masses. The dogs suffering from splenic HSA usually have no specific clinical signs which resulted in the limitation of early detection and diagnosis. Therefore, the earlier HSA detection the more success of HSA treatment is. Currently, there are a few studies about the expression of specific protein or tumor marker for splenic HSA. This research aimed to investigate the serum angiopoietin-2 (Ang-2) concentration in dogs with splenic HSA. Blood samples without anticoagulants were collected from 40 dogs with splenic abnormalities and 10 healthy female dogs control presented for routine ovariohysterectomy (OVH); prior and 10 days after splenectomy or OVH, respectively. Serum were separated after centrifugation and used for Ang-2 analysis by ELISA. The Ang-2 concentration was performed by compared with the standard curve. In addition, 40 splenic masses were sent for histopathological evaluation of the microscopic features. When HSA was diagnosed, the growth pattern and other abnormalities of spleen were investigated. The results showed that serum Ang-2 level was not significantly related to between: (1) the control dogs and the dogs with splenic abnormalities, either prior or after surgery, (2) the dogs with and without splenic tumors, (3) the dogs with benign and malignant splenic tumors, (4) the control dogs and the dogs with HSA, and (5) different staging of HSA ($P>0.05$). However, there was significantly higher serum Ang-2 level in dogs with hemangioma when compared with the control group and the HSA suffering dogs ($P<0.05$). In conclusion, it can be assumed that serum Ang-2 expression was not a specific tumor marker for canine splenic HSA.

Department:	Veterinary Surgery	Student's Signature	.....
Field of Study:	Veterinary Surgery	Advisor's Signature	.....
Academic Year:	2015	Co-Advisor's Signature	.....

ACKNOWLEDGEMENTS

My thesis could not be completed without the kind supports from many people. First of all, I am deeply grateful to my advisor Asst. Prof. Dr. Sumit Durongphongtorn who gave me knowledge and critical suggestions throughout the time. I also would like sincerely thank to my co-advisor Assoc. Prof. Dr. Somporn Techangamsuwan for sacrificing her time on precious guidance and invaluable education. My gratitudes also go to all thesis committees (Prof. Dr. Marissak Kalpravidh, Assoc. Prof. Dr. Nopadon Pirarat and Assoc. Prof. Preenun Jitasombuti) and my instructors Assoc. Prof. Dr. Achara Tawatsin for statistical suggestions and discussions to improve my thesis. I would especially thank Asst. Prof. Pasakorn Brikshavana who inspired me in veterinary surgery at the beginning. I would like to express my deep appreciation to Assoc. Prof. Dr. Athipoo Nuntaprasert and Dr. Vo Phong Vu Anh Tuan for teaching me the ELISA laboratory and analysis. The thankfulness also extends to all staff from Department of Veterinary Surgery for helping me during operation, Department of Pathology for great assistance in histopathology, the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University including the owners of the dogs participating in this research.

This research study is financially supported by the Scholarship from the Graduate School, Chulalongkorn University to commemorate the 72nd anniversary of his Majesty King Bhumibala Aduladeja and the 90th anniversary of Chulalongkorn University fund (Ratchadaphiseksomphot Endowment Fund).

Finally, I would like to cordially thank my beloved family for their encouragement to keep me moving forward along the academic career path.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

CONTENTS

Page

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Page

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

LIST OF TABLES

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

LIST OF FIGURES

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

CHAPTER I
INTRODUCTION

Importance and Rationale

Hemangiosarcoma (HSA) is a malignant neoplasm of vascular endothelial origin. It is mostly found in the spleen, the right atrium and the skin of dogs (Locke and Barber, 2006). It is accounted for 45-51% of splenic malignancies (Spangler and Kass, 1997). The HSA is mostly evident in middle-aged to older animals. The predisposing breeds are German shepherds (Oksanen, 1978), Golden retrievers, and Labrador retrievers (Schultheiss, 2004).

The diagnostic techniques are limited in HSA-bearing dogs. Blood profiles such as anemia, leukocytosis, thrombocytopenia, mild elevated liver enzymes, hypoalbuminemia, hypoglobulinemia, and common physical examination including pale mucous membrane with delayed capillary refilling time, poor pulse quality, and other clinical signs are used for diagnosis of canine splenic HSA. For the preventive medicine, diagnosis is usually done by routine abdominal ultrasonography, radiography, contrast-enhanced computed tomography (CT-scan) and magnetic resonance imaging (MRI). However, canine splenic HSA metastasizes aggressively and rapidly. Frequently, this malignancy is detected at the late stage of disease resulting in a short survival time after the treatment either by surgery or chemotherapy.

As tumor growth and metastasis have been associated with angiogenesis, the formation of new blood vessels plays essential role in tumors progression and metastasis. Angiogenesis is a complex cascade regulated by pro- and antiangiogenic factors and is essential for normally physiological processes and pathologic state of tumors. The growth factor and cytokines that involve in angiogenesis include vascular endothelial growth factor (VEGF), angiopoietins (Ang), fibroblast growth factor (FGF), transforming growth factor (TGF) and tumor necrosis factor (TNF). Moreover, there are several important factors that control vessel formation. Many studies have investigated the immunohistochemical characteristic of canine vascular tumors including von Willebrand's factor (von Beust et al., 1988), CD31 (PECAM-1; platelet endothelial cell

adhesion molecule-1)(Ferrer et al., 1995), vimentin (Moore et al., 1989b) and other biomarkers. Besides, there is a tight junction namely claudin-5, which is used as an immunohistochemical marker for distinguishing canine tumor of vascular endothelial origin (Jakab et al., 2009). In dogs with splenic HSA, the significantly increased plasma concentration of VEGF-A was noted when compared with that in healthy dogs (Clifford et al., 2001). In human, the increased VEGF concentration in serum and plasma of patients indicates a poor prognosis. As reported by Brown et al. (2000), angiopoietin-2 (Ang-2) marker expression was higher in human cutaneous angiosarcomas when compared with normal tissue (Brown et al., 2000). Accordingly, Goritz et al. (2013) reported that Ang-2 may be useful as an immunohistochemical marker for canine neoplastic endothelial cell in splenic HSA (Goritz et al., 2013).

This study aimed to investigate a correlation between serum Ang-2 concentration in canine splenic HSA comparing with that in healthy dogs. Furthermore, serum Ang-2 concentration will be evaluated at different histopathological stages of canine splenic HSA. This factor might serve as a potential diagnostic screening marker and a prognostic indicator for canine splenic HSA.

Objectives of study

1. To measure angiopoietin-2 concentrations in dogs with splenic mass compared with healthy dogs.

2. To investigate a correlation between serum angiopoietin-2 expression in canine splenic HSA and its different stages.

3. To investigate a relationship between survival time in canine splenic HSA after splenectomy and its different growth patterns.

Research questions

1. Does the angiopoietin-2 significantly increase expression in HSAs groups compared with others?

2. Does the angiopoietin-2 play a potential diagnostic screening marker and prognostic indicator for canine splenic HSA?

3. Does the angiopoietin-2 levels correlate in different stages of canine splenic HSA?

4. Is the survival time different in growth patterns of splenic canine splenic HSA?

CHAPTER II
LITERATURE REVIEW

Hemangiosarcoma

Hemangiosarcoma (HSA) is a malignant neoplasm of endothelial origin that is frequently found in dogs. Additionally, HSA was reported in other species including cattle, sheep, cat and horse (Goldschmidt and Hendrick, 2008). HSA commonly occurs in the spleen, right atrium, subcutis and others, such as liver, lung, aorta, kidney, oral cavity, muscle, bone, intestine, urinary bladder, prostate, vulva-vagina, tongue, conjunctiva and peritoneum (Withrow and MacEwan, 2001). Cutaneous HSA often arise on the prepuce and ventral abdomen. It has been related to ultraviolet light exposure in dogs (Ward et al., 1994). HSA in human has been shown with exposure to insecticides and irradiation (Paik and Komorowski, 1976). As spleen is a major hematopoietic organ, splenic HSAs account for a serious and fatal condition when occurred. Besides frequently seen in old-aged dogs, the German shepherd, Golden and Labrador retrievers are predisposing breeds for HSA (Oksanen, 1978; Christensen et al., 2009). By histopathological study, splenic masses were diagnosed as non-malignant diseases (47%) and malignant splenic neoplasms (53%). Of these, HSA is the most frequent histological diagnosis (73%) (Eberle et al., 2012).

The clinical signs of dogs with HSA are pale mucous membrane, delayed capillary refilling time, tachycardia, asymptomatic abdominal swelling, fluctuation, poor pulse quality and acute collapse. Diagnostic techniques for HSA include with complete blood count, serum biochemistry, coagulation profiles, imaging, and echocardiography. Blood results are anemia, leukocytosis, thrombocytopenia, mild elevated liver enzymes, hypoalbuminemia, and hypoglobulinemia.

HSA metastasizes through transabdominal or hematogenous implantation and the most common metastatic sites are the liver, omentum, lung and mesentery (Withrow and MacEwan, 2001). Prognosis and treatment for HSA are varied by primary sites of tumors. Surgery and chemotherapy can extend survival time after splenectomy (Clifford et al., 2000; Goritz et al., 2013). Survival time is shortened by the rupture of

splenic lesion leading to extensive internal hemorrhage and the presence of metastatic disease (Brown et al., 1985). The median survival time of canine splenic HSA after surgery and chemotherapy is lesser than six months (Sorenmo et al., 2000). Accordingly, the prognosis is definitely poor.

There are three histological growth patterns of canine splenic HSA consisting of cavernous growth pattern which are lined by differentiated neoplastic endothelial cells, capillary growth pattern which included irregular vascular channels, and solid growth pattern which is characterized by solid sheets (Maxie, 2007). The morphology of tumor endothelial cells in HSA is spindle cells with round, pleomorphic or oval nuclei, prominent nucleoi, basophilic and vacuolated cytoplasm with a high nuclear to cytoplasmic (N:C) ratio (Bertazzolo et al., 2005). Dogs with HSA will be classified as stage I: tumor confined to one organ without rupture; stage II: tumor rupture with or without regional lymph node involvement and no evidence of distant metastasis; and stage III: ruptured tumor or tumor invading another structure with distant metastases (Withrow et al., 2013).

Canine HSA has been studied in molecular basis to find a specific tumor marker. Several animal studies have investigated the immunohistochemical characteristic of canine HSA including von Willebrand's factor (von Beust et al., 1988), CD31 (PECAM-1; platelet endothelial cell adhesion molecule-1) (Ferrer et al., 1995), and vimentin (Moore et al., 1989a). There is also a study of the tight junction, claudin-5, which is an immunohistochemical marker for canine HSA (Jakab et al., 2009). Additionally, as reported by Goritz et al. (2013), angiopoietin-2 (Ang-2) and vascular endothelial growth factor A (VEGF-A) which are beneficial in canine HSA diagnosis.

Several reports have studied by using enzyme-linked immunosorbent assay (ELISA). For example, serum ferritin concentration is elevated in all cases of HSA, lymphoma, hemangioma, and hematoma. Thus, increased serum ferritin concentration is not specific for splenic HSA diagnosis (Chikazawa et al., 2013). Serum vascular endothelial growth factor (VEGF) has been also study in dog with HSA. Serum VEGF levels are significantly elevated in canine HSA compared with healthy dogs (Clifford et al., 2001). Nevertheless, it is not different from other splenic masses. Therefore, serum

VEGF concentration is not a potential diagnostic marker for canine HSA as well (Frenz et al., 2014).

As reported by Kirby et al. (2011), collagen XXVII alpha 1 may be useful for canine HSA diagnosis in advance stage (Kirby et al., 2011). Furthermore, the big endothelin-1 (big ET-1) has been investigated by using ELISA. Serum concentration of big ET-1 in canine HSA is significantly higher than other dogs. Serum concentration of big ET-1 in dog with splenic HSA is reduced after splenectomy. However, serum concentration of big ET-1 are also increased in pulmonic hypertension, renal disease, some tumors and inflammatory response (Fukumoto et al., 2015). Among them, one essential molecule, angiopoietin-2 (Ang-2), will be investigated in this study.

Angiogenesis

The vascular system is generated from endothelial cells, perivascular cells and smooth muscle cells. Angiogenesis is cooperation of endothelial cells and supporting cells during vessel sprouting, vessel maturation and generating branched network of new blood vessels.

Physiological angiogenesis

The physiological angiogenesis in vascular formation consists of three major cellular processes. There are vasculogenesis, angiogenesis and arteriogenesis. Vasculogenesis is the system of developing embryonic blood vessels from progenitor cells (Risau and Lemmon, 1988). A process of the formation of arteries and veins is angiogenesis. Development of arteries and veins from pre-existing vessels occurs through different mechanisms. For example, the splitting of vascular tube via intussusception angiogenesis or the new blood vessels sprout from pre-existing vessels. However, the sprouting of new blood vessels is commonly applied in a process of angiogenesis. The angiogenic signals activate quiescent endothelial cells. Activated endothelial cells contribute the separation of pericytes from the capillary and modify the proteolytic balance of degradation of the basement membrane. Then, endothelial cells proliferate and expand filopodia to motile. These endothelial cells are called tip

cells. After that, stalk cells extend filopodia to generate a lumen. Proliferative stalk cells promote the elongated formation. Finally, tip cells combine with cells from nearby sprouts. The interconnection of vessels is generated. Arteriogenesis is process of increasing diameter and provide stability. The perivascular cells and smooth muscle cells are coated newly vascular tubes (Gerhardt et al., 2003). In conclusion, physiological angiogenesis proceeds by activated quiescent endothelial cells, vascular destabilization, endothelial cell proliferation, migration, sprouting of new blood vessels and anastomosis. Then, the resolution phase is finalized with reducing endothelial cell proliferation and stabilization of the new blood vessels.

Pathological angiogenesis

The pathological angiogenesis is commonly found in the loss of endothelium's quiescent state, including inflammation, vasculopathies and cancer. The process is similar to physiological angiogenesis, but it generates an excessive vascular network and fails in resolution phase (Fagiani and Christofori, 2013). Many crucial molecules in angiogenic processes are shared by inflammation such as VEGF-A and angiopoietins (Ang). Thus, it implies to intimately relate between angiogenesis and inflammation (Fiedler and Augustin, 2006).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Tumor angiogenesis

The tumor angiogenesis consists of malignant tumor cells, pericytes, fibroblastic cells, inflammatory cells, and extracellular matrix surrounding cells. This system requires a variety of angiogenic factors to generate signals. The important growth factors in the system are VEGF and Ang. Signaling is established from their endothelial receptors such as fetal liver kinase-1 (FlK-1) and TiE-2 receptor. These stimuli from angiogenic factors are responsible for cell proliferation, migration, and survival of activated endothelial cells. Moreover, these signaling are able to control the maturation and maintenance of the vessels (Shim et al., 2007).

In cancer, the tumor cells outgrowth is faster than the generation of new blood vessels that support them. This finding leads the tumor cells becoming hypoxia. The

balance between pro-angiogenic and anti-angiogenic molecules is altered. Pro-angiogenic molecules are increased from both cancer cells and stromal cells. This angiogenic switch stimulus the expression of angiogenic factors, including VEGF-A, platelet-derived growth factors (PDGFs), lysophosphatic acid (LPA), and Ang (Hanahan and Weinberg, 2011). Sprouting and maturation of endothelial cells depend on Ang and VEGF-A activities including interaction between endothelial cells, perivascular cells and stromal cells (Eilken and Adams, 2010). The formation of new blood vessels plays essential roles in tumor progression and metastatic disease. Thus, tumor endothelial cells show an abnormal phenotype and morphological changes. Tumor endothelial cells from highly metastatic neoplasms have more pro-angiogenic phenotypes than those from low metastatic neoplasms (Hida et al., 2013). Moreover, tumor neovascularization involves with pluripotent progenitor cells including endothelial progenitor cells, hematopoietic stem cells and mesenchymal stem cells that are attracted into the lesion (Furuya et al., 2009). Disruption of the angiogenic regulation by inducing the release of angiogenic factors from local cells and suppressing anti-angiogenic factors can cause tumors (Folkman, 1995).

The molecular basis of angiogenesis has indicated a number of growth factor receptor pathways which promoting tumor neovascularization. The growth factor and cytokines that involved angiogenesis include VEGF, Angiopoietins, fibroblast growth factor (FGF), transforming growth factor (TGF) and tumor necrosis factor (TNF). Besides, there are remained several important factors that control vessel formation.

Tie receptors

Tie receptors are mainly expressed in the vascular and lymphatic endothelial cells including hematopoietic cells (Mazzieri et al., 2011). These receptors are identified as Tie-1 and Tie-2 tyrosine kinase receptors. Structure of Tie receptors consists of two immunoglobulin-like domains at the N-terminus, which are connected with three epidermal growth factor-like domains and another immunoglobulin-like domain, and are linked to three fibronectin type III-like domains. The C-terminus contains tyrosine kinase domain (Figure 1). In summary, the extracellular N-terminal domain is a region

of angiopoietins attachment and the intracellular C-terminal domain involves signal transduction (Fagiani and Christofori, 2013).

Tie-1 receptor

Tie-1 receptor has no specific ligand but it is able to heterodimerize with Tie-2 receptor. Tie-1 receptor binds indirectly to angiopoietins. It makes a complex form with angiopoietins and Tie-2. After a complex formation, the cytoplasmic tyrosine residues are phosphorylated. Tie-1 has weaker kinase activity than Tie-2 (Saharinen et al., 2005). Tie-1 receptor is not necessary for angioblast differentiation in early stage of angiogenesis, but it promotes vascular growth and endothelial cell proliferation in late phase (Partanen et al., 1996). However, the function of Tie-1 receptor binding angiopoietins remains unclear.

Tie-2 receptor

Tie-2 receptor is specific for angiopoietins such as angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2). Tie-2 receptor binds angiopoietins directly, and then generates strong kinase activity. Tie-2 receptor is not essential for the formation of new blood vessel, but it supports endothelial cell proliferation and maintenance of vasculature (Dumont et al., 1994). Both Tie-1 and Tie-2 receptors play essential roles in a formation of microvasculature during late stage of embryogenesis. In addition, both Tie receptors are also important for adult angiogenesis (Puri and Bernstein, 2003).

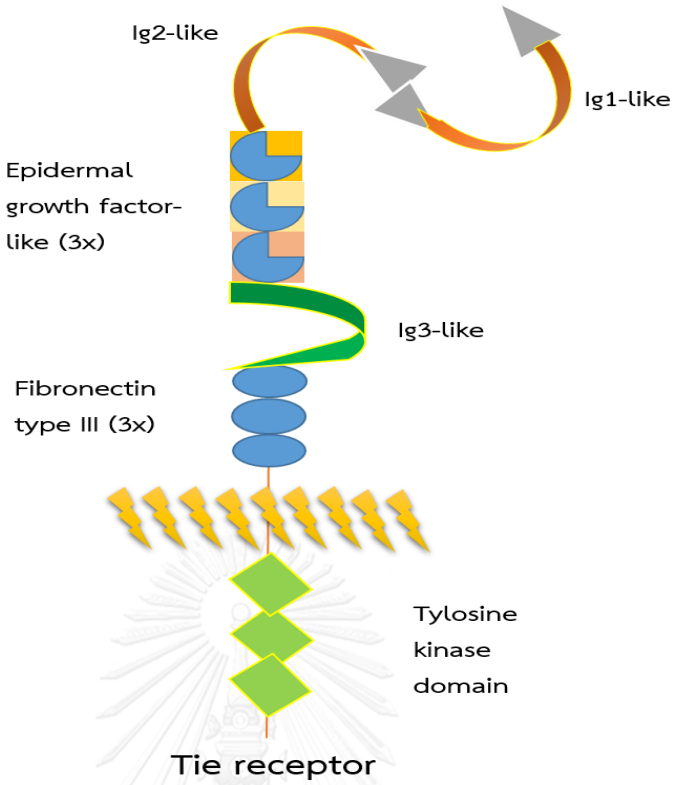


Figure 1 Molecular structure of Tie receptors.

Angiopoietins

Angiopoietins, the multimeric ligands of Tie-2 receptor, accounts for the major pathway involving in the control of blood vessels development and stability. Angiopoietins are a glycoprotein with a molecular weight of approximately 70 kDa. There are four distinct angiopoietin: angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), angiopoietin-3 (Ang-3) and angiopoietin-4 (Ang-4) (Fagiani and Christofori, 2013). Angiopoietin proteins comprise an amino-terminal domain, followed by a coiled-coil domain that promotes multimerization, and carboxy-terminal fibrinogen homology domain that contains the binding sites for Tie-2 receptor (Figure 2) (Davis et al., 2003). Only multimeric structure of angiopoietins is required for Tie-2 activation. Additionally, angiopoietins also bind Tie-2 receptor to activate their receptors and heterodimerize Tie1/Tie-2 receptor (Saharinen et al., 2005).

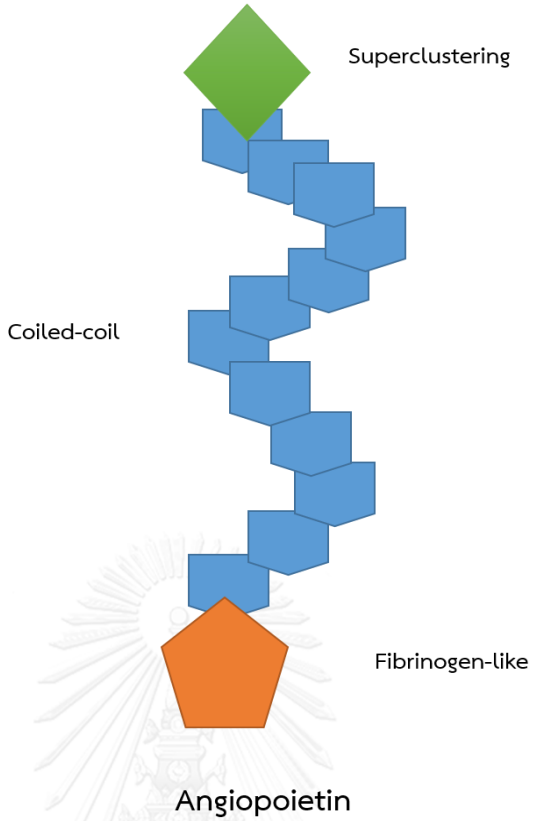


Figure 2 Molecular structure of angiopoietin ligands.

Angiopoietin-1

Angiopoietin-1 (Ang-1) is expressed by perivascular cells. Ang-1 is an activating ligand for Tie-2 receptor. These form leads to phosphorylation and signal transduction. Ang-1 and Tie-2 signal is necessary for endothelial cell proliferation, vascular maturation, and survival of activated endothelium. Moreover, the ability to regulate angiogenesis depends on signal from VEGF and Ang-1. VEGF induces vascular sprouting and Ang-1 is responsible for vessel maturation and remodeling. The combination of VEGF and Ang-1 activity is able to induce highly differentiated blood vessels (Thurston et al., 1999).

Angiopoietin-2

Angiopoietin-2 (Ang-2) is stored in endothelial cell's secretory granules called Weibel-palade bodies and will be released in inflammatory responses (Fiedler et al., 2004). Therefore, Ang-2 is significantly expressed in endothelial cells and its increased

expression is noted when the inflammation and angiogenesis occurred. The new blood vessel formation influences in dissemination of tumor cells to distant site. The angiogenesis is crucial for supply of oxygen, nutrients, growth factors, hormones and proteolytic enzymes (Moreira et al., 2007). The Ang-Tie system promotes angiogenic remodeling of blood vessels during chronic inflammation. Moreover, Ang-2 has an influence in tumor vessel sprouting. Its expression is increased in tumor-associated endothelium (Thomas et al., 2009) and its high levels correlate with malignancy in various cancer types, such as acute myeloid leukemia, chronic lymphocytic leukemia, multiple myeloma, breast cancer and melanoma (Hu et al., 2009; Lauri et al., 2013). Generally, advanced tumor stage in a variety of human cancer is associated with increasing tumor vascularization and tumor expression of proangiogenic factors.

The serum concentration of Ang-2 has been elevated in various human cancers such as cervical cancer (Kopczyńska et al., 2009), colon cancer (Engin et al., 2012), gastric cancer (Etoh et al., 2001), squamous cell carcinoma (Hawighorst et al., 2002), breast cancer (Sfiligoi et al., 2003), melanoma (Helfrich et al., 2009) and colorectal cancer (Wang et al., 2015). Moreover, elevated serum Ang-2 levels associate with vascular dysfunction in various pathological conditions. For example, disseminated intravascular coagulopathy (Wada et al., 2012), sepsis (van der Heijden et al., 2009), acute liver failure (Hadem et al., 2012) , acute lung injury and traumatic patient (Ganter et al., 2008). Therefore, the increased expression of Ang-2 in human cancers and disease might be related with tumor vascularization.

As reported by Brown et al. (2000), a higher expression of Ang-2 has been found in human cutaneous angiosarcomas when compared with normal tissue. While Goritz et al. (2013) reported that Ang-2 may be considered as a useful immunohistochemical marker for canine neoplastic endothelial cell in splenic HSA.

Angiopoietin-3

Angiopoietin-3 (Ang-3) is less well-studied. Ang-3 is not able to phosphorylate Tie-2 receptor. There is an antagonistic relationship between Ang-1 and Ang-3 on Tie-2 receptor (Valenzuela et al., 1999). Nevertheless, Ang-3 has ability to activate Tie-2 receptor in mouse (Lee et al., 2004).

Angiopoietin-4

Angiopoietin-4 (Ang-4) is found in human lung and phosphorylates Tie-2 receptor (Valenzuela et al., 1999). The function of Ang-4 is remained unclear.

Ang-Tie system

Ang-Tie system is important for vessels remodeling and maturation during embryogenesis and adult vessel angiogenesis (Thurston, 2003). Ang-1 is mainly expressed in perivascular cells such as tumor cells, pericytes, fibroblasts and smooth muscle cells. The expression of Ang-1 is elevated in pericytes and smooth muscle cells during hypoxia (Park et al., 2003). While Ang-1 is binding Tie-2 receptor (Figure 3), it leads to auto-phosphorylate at tyrosine residues. Intracellular signaling pathways are generated, especially phosphoinositide-3 kinase (PI3K) pathway which supports endothelial survival. Thus, Ang-1 activated Tie-2 receptor involves the survival of endothelial cells and maintenance of the vessels. Ang-2 is predominantly expressed in endothelium. The production of Ang-2 is regulated by several growth factors and tissue hypoxia. Ang-2 is elevated during activation of endothelial cells. However, Ang-2 is a partial agonist to Tie-2 receptor by displacing active Ang-1 from their receptors (Yuan et al., 2009). Thus, regulation of the angiogenesis and homeostasis depends on the ratio of Ang-1 to Ang-2.

CHULALONGKORN UNIVERSITY

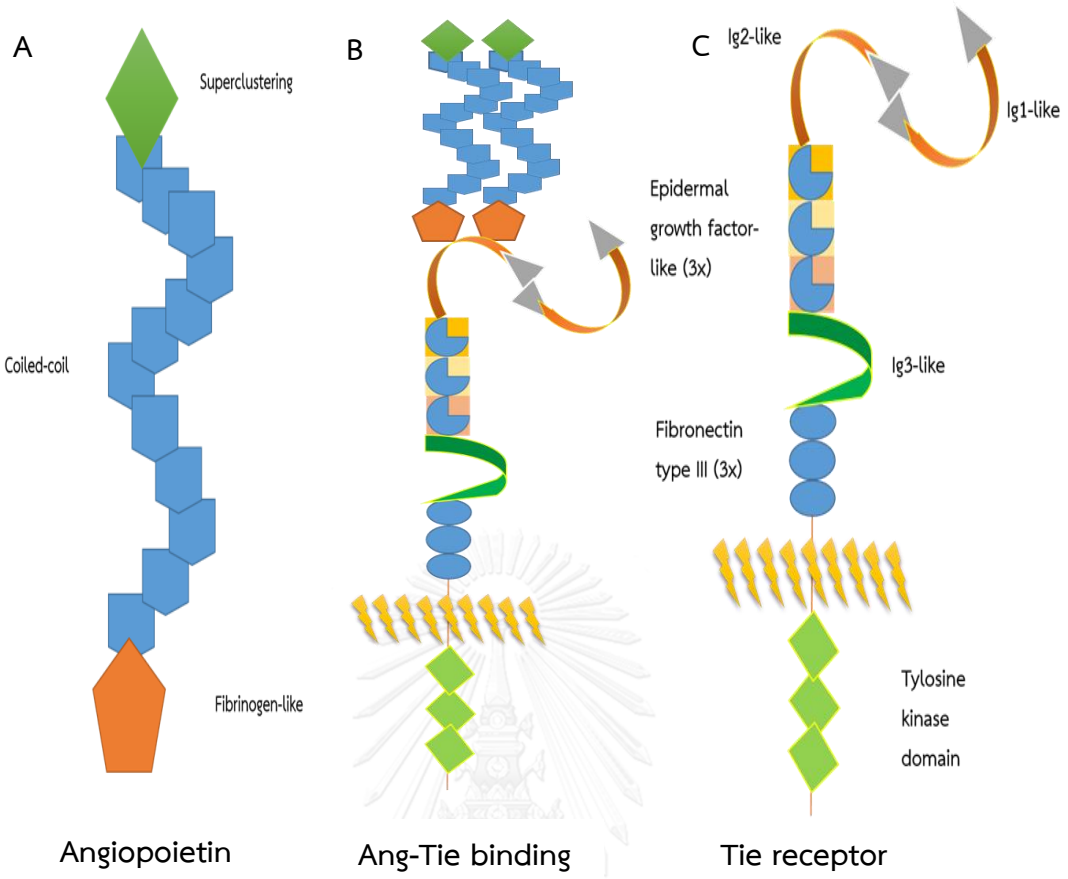


Figure 3 Molecular structure of angiopoietin and Tie receptor. (A) Angiopoietin molecule. (B) Angiopoietin binds to Tie-2 receptor. (C) Tie receptor.

Chulalongkorn University

The role of Ang-Tie signaling system

1. The role of Ang-Tie signaling system in blood vessels development

The Ang-Tie system plays an important role in blood vessels development which is necessary to vascular sprouting, vessel remodeling, vascular maturation, and activate quiescent endothelial cell (Hanahan, 1997). Ang-1-Tie-2 signaling system is responsible for vascular stability. Ang-1 inhibits vascular permeability induced by VEGF. It promotes adhesion molecule at junctional structures of endothelium. As reported by Fiedler et al. (2004), the ratio Ang-1/Ang-2 is decreased by releasing of Ang-2 from Weibel-palade bodies. This results in suppression of Ang-1 signaling and leads to vascular destabilization. Thus, the Ang-2 acts as Ang-1 antagonist to Tie-2 receptor (Fagiani and Christofori, 2013).

2. The role of Ang-Tie signaling system in inflammation

The Ang-2 signaling is important for vascular remodeling during inflammation (Tabruyn et al., 2010), whereas Ang-1 activity is an anti-inflammation. Ang-1 suppresses endothelial transmigration and leukocyte adhesion. It leads to decrease inflammatory response. Therefore, Ang-1 has effects against Ang-2 during inflammation. Ang-2 supports inflammatory mediators but Ang-1 is limited by Ang-2/Tie-2 activation.

3. The role of Ang-Tie signaling system in tumor angiogenesis

The role of Ang-Tie system in tumor angiogenesis is initiated by activating endothelial cells which are able to produce Ang-2. The activation of Ang-2 causes endothelial destabilization and regression of vessels. It leads to dissociation of vessels and apoptosis of endothelial cells, resulted in hypoxia condition in tumor. Then, VEGF-A is up-regulated in order to induce tumor angiogenesis. Finally, Ang-Tie signaling system combines with VEGF-A to promote vascular formation in tumor (Fagiani and Christofori, 2013). Thus, Ang-2 may be a Tie-2 agonist in tumor angiogenesis. Circulating Ang-2 also promotes metastasis (Holopainen et al., 2012). Additionally, Ang-1 limits tumor growth by supporting vascular integrity. This effect of Ang-1 may be link to anti-inflammation function (Tadros et al., 2003). In summary, both Ang-1 and Ang-2 are crucial for promoting tumor growth. The abnormal vascularization results from imbalance of Ang-1, Ang-2 and VEGF during tumor angiogenesis.

In conclusion, the Ang-Tie system plays important role in neovascularization during inflammation and tumor angiogenesis. Ang-1 is stabilized blood vessels and Ang-2 promotes vascular remodeling. The activated endothelial cell releases Ang-2 from Weibel-palade bodies. The high levels of Ang-2 induce endothelial cell destabilization and expression of VEGF. These actions promote formation of tumor blood vessels.

CHAPTER III
MATERIALS AND METHODS

Animals

This study consisted of 40 dogs with splenic masses and 10 healthy dogs as a control group. The data based on medical records, biopsy results and serology analysis. The signalments (age, sex, and breed), clinical outcome, blood profiles, imaging diagnosis, histopathological results, the date of operation (splenectomy), the date of death and survival time were recorded systematically. After splenectomy, details of splenic masses or lesions were collected and tissues were submitted to Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University for histopathological study. Moreover, blood samples were withdrawn from cephalic or saphenous veins. Serum were separated and stored in aliquot at -80°C until analysis. All experimental protocols were approved by the Chulalongkorn University Animal Care and Use Committee (No. 11310088).

Sample collection

Animals conducted in this study were collected during 2015-2016 from the Small Animal teaching hospital, Faculty of Veterinary Science, Chulalongkorn University in Thailand. The owners were informed according to the regulation of the Chulalongkorn University Animal Care and Use Committee (No. 11310088). The dogs were divided into two groups as follows:

Group 1 – Control group

Ten dogs that come for routine ovariohysterectomy (OVH) were confirmed as healthy based on a physical examination, blood profiles and abdominal ultrasonography. Blood was taken before and 10-days after OVH. Three-ml blood samples were withdrawn and collected in plain tube without anticoagulant. Whole blood samples were allowed to clot at room temperature for 30 minutes. After centrifugation at 1,000g (or 3,000 rpm) for 15 minutes, serum was collected and

transferred into a new 1.5-ml Eppendorf tube. Samples were stored in aliquot at -80°C until serology analysis.

Group 2 – Splenic mass group

Forty dogs with various splenic masses based on histopatholgical results were included composing (i) splenic non-tumor lesions such as hematoma, splenic nodular hyperplasia, splenitis, splenic infarction and splenic congestion, (ii) splenic benign tumors such as hemangioma and histiocytoma, and (iii) splenic malignant tumors such as HSA, HSA with metastatic adenocarcinoma, HSA with metastatic melanoma, HSA with metastatic sertoli cell tumor, lymphoma, malignant fibrous histiocytic sarcoma, plasma cell tumor, mast cell tumor metastasis to spleen, granulosa cell tumor metastasis to spleen. Subsequently, the splenic mass group was classified into six groups which were non-tumor, tumor, non-malignant tumor, malignant tumor, hemangioma and HSA. Each group had inclusion and exclusion criteria as followed:

1. Non-tumor group

This group consisted of hematoma, splenic nodular hyperplasia, splenitis, splenic infarction and splenic congestion. The inclusion criteria were a dog with non-tumor diagnosis and without mass in other areas of body. The exclusion criterion was other tumors in spleen.

2. Tumor group

Tumor group consisted of HSA, HSA with adenocarcinoma, HSA with melanoma, HSA with metastatic sertoli cell tumor, lymphoma, malignant fibrous histiocytic sarcoma, splenic plasma cell tumor, mast cell tumor metastasis to spleen, granulosa cell tumor metastasis to spleen, hemangioma and histiocytoma. The inclusion criteria were all benign and malignant splenic tumors included metastatic tumor to spleen. The exclusion criterion was non-splenic tumor.

3. Non-malignant tumor group

Benign splenic tumor in this study consisted of hemangioma and histiocytoma. The inclusion criterion was non-malignant splenic tumor without tumor in other organs. The exclusion criteria were malignant splenic tumor and non–tumor splenic mass including other cancer in body.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

4. Malignant tumor group

This group contained HSA, HSA with adenocarcinoma, HSA with melanoma, HSA with sertoli cell tumor, lymphoma, malignant fibrous histiocytic sarcoma, splenic plasma cell tumor, mast cell tumor metastasis to spleen and granulosa cell tumor metastasis to spleen. The inclusion criterion was all malignant tumor included metastatic tumor to spleen. The exclusion criterion was non-splenic tumor and benign splenic tumor.

5. Hemangioma group

This group consisted of only splenic hemangioma. The inclusion criterion was splenic hemangioma without other cancers.

6. HSA group

The inclusion criterion for HSA group was a dog with only splenic HSA. Whereas, the splenic HSA with metastasis of other cancer was the exclusion criteria in this group. Thus, this study had 13 dogs with splenic HSA. Among them, three dogs with other metastatic cancers were excluded from this group.

All dogs in this group were splenectomized following recommendation from certified veterinarians as a part of treatment. Serum was collected, as mentioned above, before and 10-days after splenectomy. During operation, clinical staging of splenic HSA was closely observed and classified into 3 stages as followed (Stephen et al., 2013):

Stage I: tumor confined to one organ without rupture.

Stage II: tumor rupture with or without regional lymph node involvement and no evidence of distant metastasis.

Stage III: ruptured tumor or tumor invading another structure with distant metastasis.

Finally, the spleens were removed. Tissues were collected and submitted to further diagnosis by histopathology.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Pathology for splenic tumor diagnosis and HSA staging

After splenectomy, selected splenic tissues composed of both lesional and normal areas were cut and fixed in 10 % neutral buffered formalin for 24-48 hours. Then, tissues were processed further for histopathological study. Paraffin-embedded tissues were serially sectioned at 4-μm thickness and stained with hematoxylin and eosin (H&E). Each section was diagnosed by certified pathologists at Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University. When HSA is diagnosed, it was further classified according to the criteria of specific histological growth pattern as followed (Goritz et al., 2013):

1. Cavernous growth pattern consisted of expanded vascular channels lining by malignant endothelial cells. This was able to describe by mainly cavernous with partly capillary or partly solid.

2. Capillary growth pattern composed of irregular vascular channels and described by mainly capillary with partly solid or partly cavernous.

3. Solid growth pattern consisted of solid sheets and described by mainly solid with partly capillary or partly cavernous

4. Mixed growth pattern consisted of all growth patterns equally.

In addition, this study investigated other pathological findings in splenic HSA, both macroscopic and microscopic findings. The macroscopic examinations revealed size, consistency, color and characteristic of splenic mass. Whereas, the other microscopic findings demonstrated necrosis, hemorrhage, hemosiderosis, hemophagocytosis, calcification, thrombosis, extramedullary hematopoiesis, fibrosis, mitotic figure, nuclear atypia, vascular space, amyloid deposition and blood cyst. When amyloid was suspected, it was confirmed by Congo red special staining. Furthermore, nuclear atypia was found in human splenic angiosarcoma (Neuhauser et al., 2000) and was applied in dogs with splenic HSA. Nuclear atypia was classified to three grades as mild, moderate and severe. Mild atypia was described by nuclei with minimal pleomorphism and hyperchromasia; moderate atypia referred to moderate pleomorphism and hyperchromasia, and severe atypia was described by nuclei with enormous pleomorphism and hyperchomasia.

Serology for Ang-2 assessment

All serum samples were measured for Ang-2 concentration by enzyme-linked immunosorbent assay (ELISA) with specific antibodies that recognized secreted canine Ang-2. Serum, stored in aliquot at -80°C, were allowed to thaw at room temperature and avoided multiple freeze-thaw cycles.

The assay was performed following the recommendations from the manufacturer's protocols (Novateinbio; Novatein biosciences, USA; catalog numbers BG-CAN10138). The sensitivity of the assay is 0.1 ng/ml. Dilution of Ang-2 was performed by mixing with assay buffer in 5-fold dilution. All serum samples were determined in duplicated and averaged.

Before starting procedure, all reagents and samples were brought to warm at room temperature (18°C-25°C) for 30 minutes. After setting standard, sample, and blank (control) wells, 50 µl of standard solution was added to each standard well, while 50 µl of sample was added to each sample well. Subsequently, 50 µl of sample diluents was added to each blank (control) well. Then, 100 µl of HRP-conjugate reagent was added to each well and incubated for 60 minutes at 37°C. The wash solution was diluted by mixing 475-ml distilled water with 25-ml wash solution. The microtiter plate was washed five times to remove unbound Ang-2. Fifty µl of chromogen solution A was then added, and followed by the addition of 50 µl of chromogen solution B into each well, The plate was gently mixed, covered to protect from light, and incubated for 15 minutes at 37°C. Finally, 50 µl of a stop solution was added to each well. The color in the wells developed from blue to yellow and the intensity of the color was measured (Figure 4). The samples were immediately read at an optical density (O.D.) of 450 nm using a microelisastripplate reader.

The results were calculated by the mean OD value for each standard and serum sample. All O.D. values were subtracted by mean value of the blank control. Then, the standard curve was generated using statistical software.

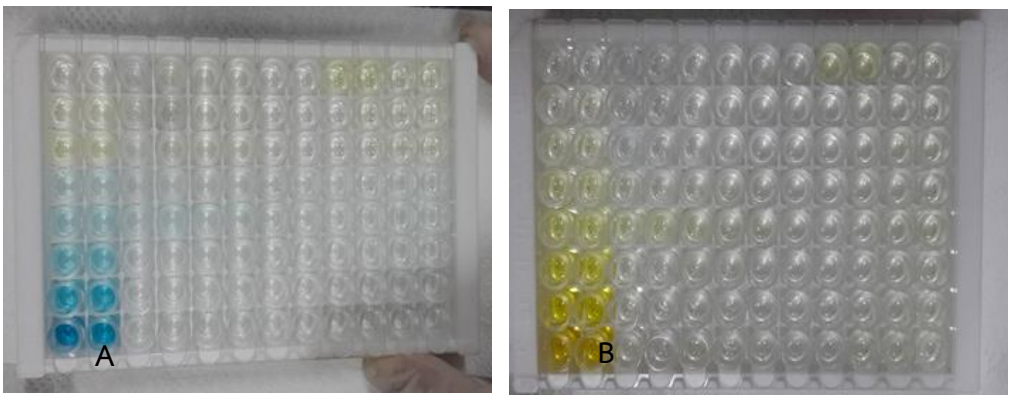


Figure 4 (A) and (B) showed the color in the wells developed from blue to yellow after adding stop solution. The intensity of the color was immediately read at an optical density (O.D.) of 450 nm using a microelisastripplate reader.

Statistical analysis

Ang-2 concentrations were not normal distribution. For data analysis, SPSS software version 22.0 (SPSS, Chulalongkorn University) was used. Statistical analysis was performed using the nonparametric test. Ang-2 levels were compared between healthy and splenic mass group, and between the HSA group and splenic mass group by Mann-Whitney U test. Ang-2 concentrations were compared between the control group, non-tumor group and splenic mass group by Kruskal-Wallis test, and between the malignant and non-malignant tumor group by Mann-Whitney U test. Additionally, The Kruskal-Wallis test was used in comparative between the control, hemangioma and HSA group. The correlation of Ang-2 concentrations in various stages of HSA was analyzed by Pearson's linear correlation. The comparison between Ang-2 concentration prior and after surgery were analyzed using the Wilcoxon test in control, hemangioma and HSA group. Ang-2 concentrations that were undetectable were set at 0.1 ng/ml. Sensitivity and specificity with 95% confidence intervals (CIs) were calculated with a cut off value of undetectable concentrations. P-values less than 0.05 were considered as significant. Survival times were analyzed by Kaplan-Meier method.

CHAPTER IV

RESULTS

A. General characteristics of the studied dogs

This study composed of 40 dogs with splenic masses and 10 healthy dogs as the control group. Of the 40 dogs with splenic masses, the large breed dogs were the majority of studied population (32.5%) including Golden retriever (n=6), Labrador retriever (n=5), Shetland sheepdog (n=1) and Siberian husky (n=1). The others were medium-sized and small-sized breed dogs which were mixed breed (n=9), Cocker spaniel (n=3), Shih Tzu (n=3), Beagle (n=2), Poodle (n=2), Thai bangkaew (n=2), Yorkshire terrier (n=1), Dachshund (n=1), Terrier (n=1), Schnauzer (n=1), French bulldog (n=1), and Bulldog (n=1). The percentage of particular breeds were presented in Figure 5.

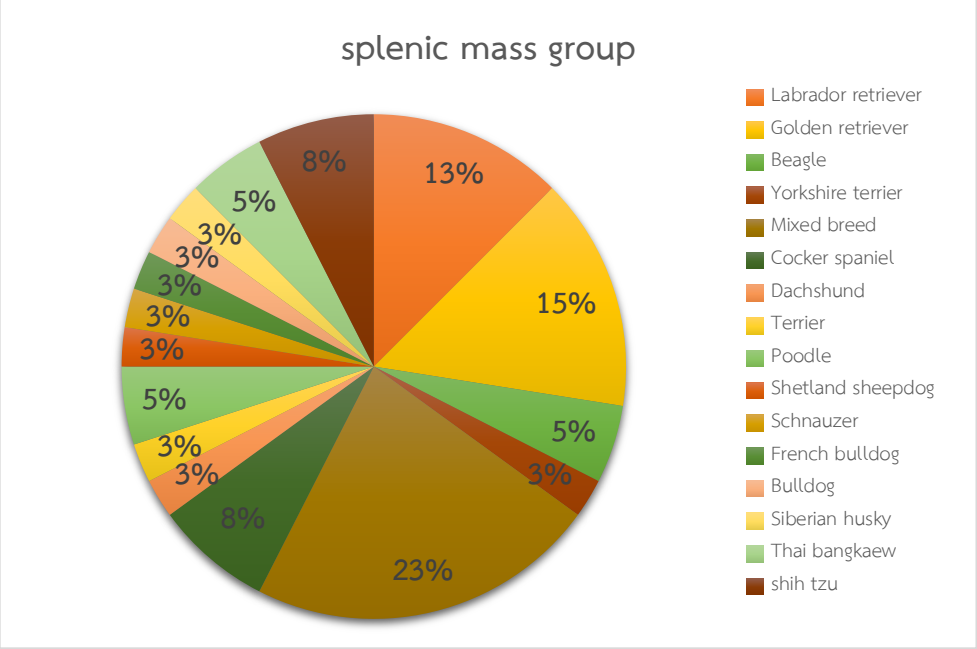


Figure 5 The percentage of breed in splenic mass group.

The mean age of dogs with splenic masses was 10.9 years (n=40, range 4-16 years, SD=2.92). Genders of 40 cases consisted of entire male (30%, n=12), neutered male (37%, n=15), entire female (15%, n=6) and neutered female (18%, n=7) (Figure 6). The ratio of male to female was 2:1.

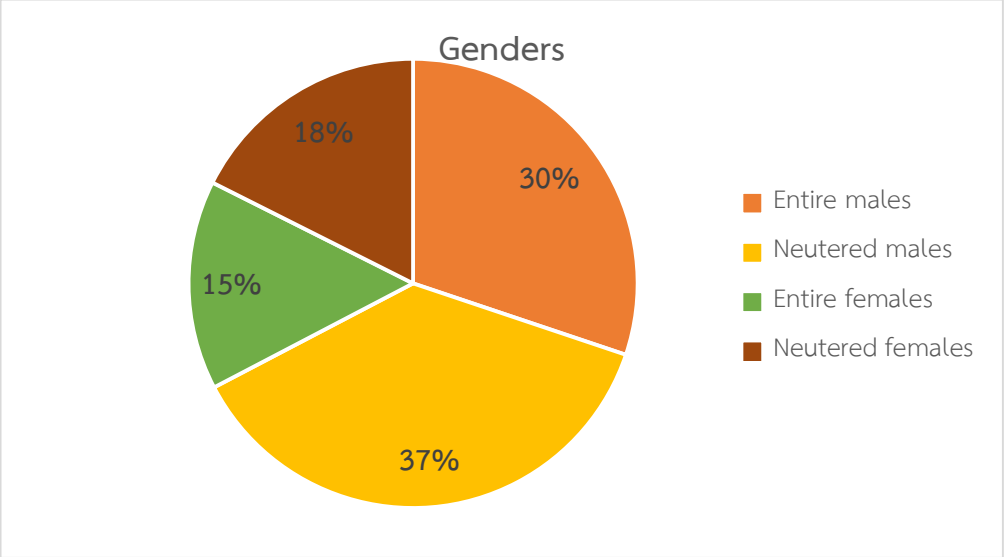


Figure 6 The percentage of genders in splenic mass group.

The control group consisted of 10 healthy dogs. The mean age of healthy dogs was 10.2 months (Range 8-12 months, SD= 1.475). There were various breed such as Pomeranian, Chihuahua, Golden retriever, Shih Tzu and mixed breed. Only female dog preparing for OVH were included.

Biopsy results of 40 dogs were reviewed on the basis of histopathological diagnosis and showed in table 1. This study found that 12 dogs contained splenic non-tumors including hematoma, splenic nodular hyperplasia, splenitis, splenic infarction and splenic congestion. The other 28 dogs possessed either splenic malignant tumor (n=21) and splenic benign tumor (n=7). The ratio of malignancy to benign was 3:1. The highest incidence of splenic malignant neoplasm was HSA (32.5%, n=13). Among them, it consisted of single tumor as HSA (n=10) and HSA with other metastatic cancers such as adenocarcinoma (n=1), melanoma (n=1), and sertoli cell tumor (n=1). Whereas the commonly found benign splenic tumor was hemangioma (15%, n=6). Considering within splenic tumor group, 61.9% of dogs was HSA (13/21) and 85.7% was hemangioma (6/7).

Table 1 The histolopathological diagnosis of 40 dogs with splenic masses

Histopathological results	**Number (%)**
Malignant tumor	
Hemangiosarcoma	13 (32.5%)
Lymphoma	4 (10%)
Splenic plasma cell tumor	1 (2.5%)
Mast cell tumor	1 (2.5%)
Granulosa cell tumor	1 (2.5%)
Malignant fibrous histiocytic sarcoma	1 (2.5%)
Benign tumor	
Hemangioma	6 (15%)
Histiocytoma	1 (2.5%)
Non-tumor	
Splenic nodular hyperplasia	3 (7.5%)
Splenitis	5 (12.5%)
Hematoma	2 (5%)
Splenic congestion	1 (2.5%)
Splenic infarction	1 (2.5%)
Total	*40 (100%)*

B. General characteristicsof the dogs with splenic HSA

The breed, age, sex and clinical staging of 13 dogs with splenic HSA in this study were presented in table 2.

Table 2 General characteristic and clinical staging of the dogs with splenic HSA

No.	Breed	Age (year)	Sex[1]	Clinical staging[2]	Survival time[3] (days)
1	Labrador retriever	10	Mc	3	56
2	Golden retriever	7	Mc	3	51
3*	Labrador retriever	12	Mc	3	57
4	Beagle	12	M	3	199
5	Golden retriever	9	M	3	0
6	Yorkshire terrier	12	Mc	1	N/A
7**	Beagle	12	Mc	2	90
8	Labrador retriever	12	Mc	2	264
9	Mixed	12	F	3	6
10	Labrador retriever	12	F	3	5
11	Mixed	14	F	3	6
12	Labrador retriever	12	M	3	73
13***	Mixed	13	F	3	9

Chulalongkorn University

[1]M, male; Mc, neutered male; F, female

[2]Stage 1,tumor confined to one organ without rupture; stage II, tumor rupture with or withoutregional lymph node involvement and no evidence of distant metastasis; stage III, ruptured tumor or tumor invading another structure with distant metastasis (Stephen et al., 2013).

* = The dog with splenic hemangiosarcoma and melanoma

** = The dog with splenic hemangiosarcoma and sertoli cell tumor

*** = The dog with splenic hemangiosarcoma and adenocarcinoma

[3]N/A, data not avaliable

The most common breed with HSA in this study were Labrador retriever (n=5, 39%), followed by mixed breed (23%, n=3), Golden retriever (15%, n=2), Beagle (15%, n=2) and Yorkshire terrier (8%, n=1).

The mean age of dogs with splenic HSA was 11.5 years (n=13, range 7-13 years, SD=1.81). The genders of the dogs with splenic HSA were found in male more than female. There were 6 neutered males, 4 entire males and 3 entire females.

Clinical staging of splenic HSA in 13 dogs was closely observed during splenectomy and classified into 3 stages according to the previous study (Withrow et al., 2013). The clinical stage 3 was frequently found (77%, n=10), followed by clinical stage 2 (15%, n=2) and clinical stage 1 (8%, n=1) (table 2).

C. Pathology of the dogs with splenic masses

Macroscopic findings

In general, the 40 spleens had both single and multiple masses and located either at head, body or tail. The size of those masses ranged from 1x1 cm to 50x70 cm. The coloration of splenic masses was dark-red with or without some white foci. The splenic surface had both irregular and smooth. The consistency of the tumors was almost firm. Furthermore, the cut surface was bulgy, dark-red color with white-yellowish necrotic tissue. Some spleens had cyst-like lesion which contained serosanguineous fluid and/or blood clot. Other lesions composed of hematomas, hemorrhage, and adhesion to other adherent organs. Moreover, the rupture of splenic tumors was found in 14 out of 40 (35%), in which comprised 9 HSA, 2 histiocytic splenitis, 1 lymphoma, 1 MCT and 1 plasma cell tumor (Table 3).

The splenic HSA had either single or multiple masses in which a prominent large mass were frequently observed. The common location was found at body and tail of spleen. The size of tumor ranged from 2x2 cm to 50x70 cm. The HSA had a dark-red color, firm consistency with irregular splenic surface. Besides, hemorrhage, necrosis and adhesion to other organs were noted. The cut surface was bulgy and dark-red in color. Moreover, there was tumor rupture in 9 out of 13 (69%) in HSA group (Figue 7).

In this study, hemangioma had a single mass with hematoma-like lesion, in which composed of blood clot. The hemangioma ranged in size from 3x5 cm to 15x30 cm. There were smooth surface and firm in consistency. All of them was not rupture. The location of tumor was various included head, body and tail. Additionally, the cut surface was bulgy and dark-red in color (Figure 8).

All splenic lymphoma had a single mass which ranged in size from 3x5 cm to 25x35 cm. The coloration of tumor had dark-red with the white plaque or intraluminal necrotic tissue. There were both smooth and irregular surface. The consistency of mass was firm. Tumor rupture was found in 1 dog (Dog no.22). Furthermore, the cut surface was firm and cystic mass contained serosanguineous exudate with necrotic tissue (Figure 9).

In addition, splenic plasma cell tumor was shown in figure 10. All of the spleen had diffusely multiple masses. There were smooth surface and firm in consistency. The cut surface was bulgy with white-red. Hemorrhage and rupture were also found. Moreover, the metastatic tumor was instanced by MCT. The spleen with MCT had multiple nodules. There was firm, dark-red in color and ranged in size 1x2 cm (Figure 11).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 3 Macroscopic findings and histopathological diagnosis of 40 splenic masses.

No.	Locations of mass at spleen	Gross lesions	Size (cm)	Colors	Consistency	Tumor rupture	Histopathological diagnosis	Metastasis
1	Body to tail	Single	50x60	Dark red	Firm	Yes	HSA	Yes
2	Body	Single	10x15	Dark red	Firm	Yes	HSA	Yes
3	Head	Multiple masses	10x15	Dark red	Firm	No	HSA and metastatic melanoma	No
4	Body	Multiple masses	10x12	Dark red	Firm	Yes	HSA	Yes
5	Body to tail	Large multiple masses	50x70	Dark red	Firm	Yes	HSA	Yes
6	Tail	Single	2x2	Dark red	Firm	No	HSA	No
7	Body	Single	4x6	Dark red	Firm	No	HSA and metastatic Sertoli cell tumor	No
8	Tail	Single	15x15	Dark red	Firm	No	HSA	No
9	Tail	Large multiple masses	5x6	Dark red	Firm	Yes	HSA	Yes
10	Body to tail	Multiple nodules with a large single mass	50x60	Dark red	Firm	Yes	HSA	Yes
11	Tail	Single	10x12	Dark red	Firm	Yes	HSA	Yes

No.	Locations of mass	Gross lesions	Size (cm)	Colors	Consistency	Tumor rupture	Histopathological diagnosis	Metastasis
12	Body to tail	Multiple nodules with a large single mass	30x50	Dark red	Firm	Yes	HSA	Yes
13	Head	Single	7x8	Dark red	Firm	Yes	HSA and metastatic adenocarcinoma	Yes
14	Body	Single	15x30	Dark red	Firm	No	Hemangioma	Yes
15	Head	Single	8x8	Dark red	Firm	No	Hemangioma	No
16	Tail	Single	8x8	Dark red	Firm	No	Hemangioma	No
17	Tail	Single	10x10	Dark red	Firm	No	Hemangioma	No
18	Tail	Single	3x5	Dark red	Firm	No	Hemangioma	No
19	Body	Single	10x10	Dark red	Firm	No	Hemangioma	No
20	Body	Single	3x5	Dark red	Firm	No	Lymphoma	No
21	Body	Single	20x25	Dark red/ white	Firm	No	Lymphoma	No
22	Tail	Single	25x35	Pale with	Firm	No	Lymphoma	No
23	Tail	Single	8x6	Dark red	Firm	Yes	Lymphoma	No
24	Tail	Single	3x3	Dark red	Firm	No	Hematoma	No

No.	Locations of mass at spleen	Gross lesions	Size (cm)	Colors	Consistency	Tumor rupture	Histopathological diagnosis	Metastasis
25	Tail	single	7x8	Dark red	Firm	No	Hematoma	No
26	Body	single	1x1	White	Firm	No	Nodularhyperplasia	No
27	Diffuse	Multiple masses	4x5	Dark red	Firm	No	Nodular hyperplasia	No
28	Tail	Single	3x4	Dark red	Firm	No	Nodular hyperplasia	No
29	Tail	Single	4x3	White	Firm	No	Malignant fibrous histiocytic sarcoma	No
30	Diffuse	Large multiple masses	15x20	Dark red /white	Firm	Yes	Splenic plasma cell tumor	Yes
31	Body	Single	20x30	Dark red /white	Firm	No	Histiocytoma	No
32	Tail	Single	5x5	Dark red	Firm	No	Splenic infarction	No
33	Body	Single	4x5	Dark red	Firm	No	Splenic congestion	No
34	Diffuse	Multiple masses	1x2	Dark red	Firm	Yes	Metastasis MCT	Yes
35	Tail	Single	10x12	Dark red	Firm	No	Metastatic granulosa cell tumor	Yes
36	Tail	Single	15x20	Dark red	Firm	No	Plamacytic splenitis	No
37	Tail	Single	6x7	Dark red	Firm	Yes	Histiocytic splenitis	No
38	Diffuse	Multiple masses	15x33	Dark red	Firm	Yes	Histiocytic splenitis	No
39	Tail	Single	2x3	Dark red	Firm	No	Suppurative splenitis	No
40	Body	Single	12x12	Dark red	Firm	No	Necrotic splenitis	No

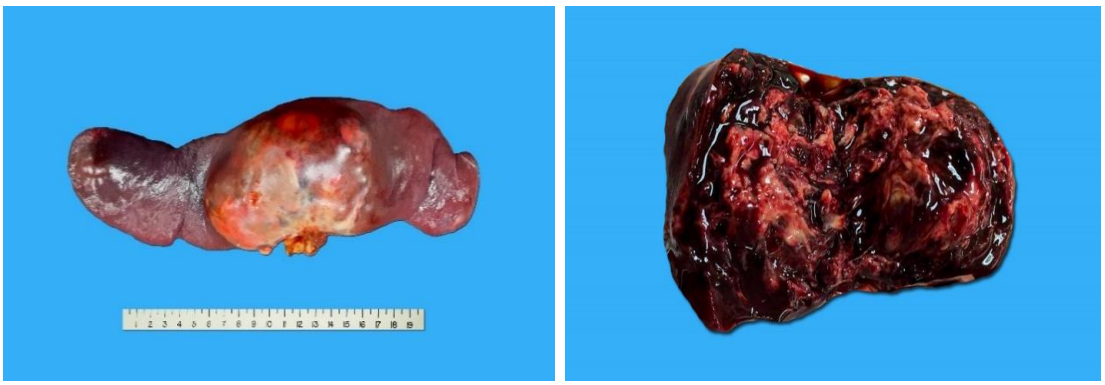
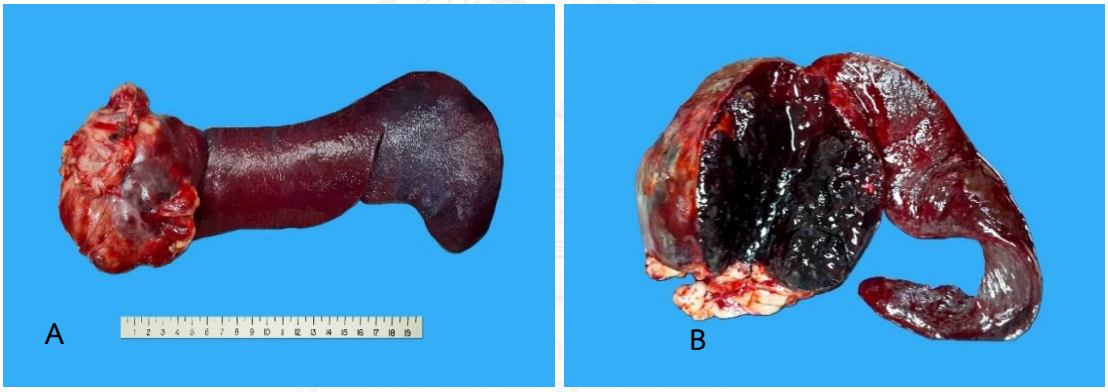


Figure 7 Canine splenic HSA (Dog no. 8). (A) The spleen with a single mass (size 15x15 cm) at body had dark-red in color and firm consistency. (B) The cut surface of the splenic HSA was bulgy and consisted of blood clot and prominent necrotic tissue.



Chulalongkorn University

Figure 8 Canine splenic hemangioma (Dog no. 17). (A) The single mass at tail, size 10x10cm, had dark-red in color and firm consistency. (B) The cut surface of splenic mass consisted of blood clot.

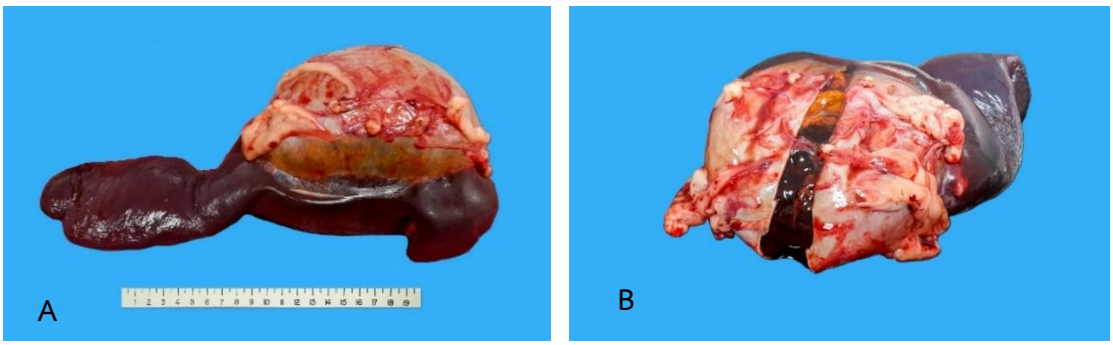


Figure 9 Canine splenic lymphoma (Dog no. 22). (A) A large single mass (size 15x15 cm) at tail had dark-red with the white plaque in color and firm consistency. (B) The splenic surface composed of necrosis and blood filled.

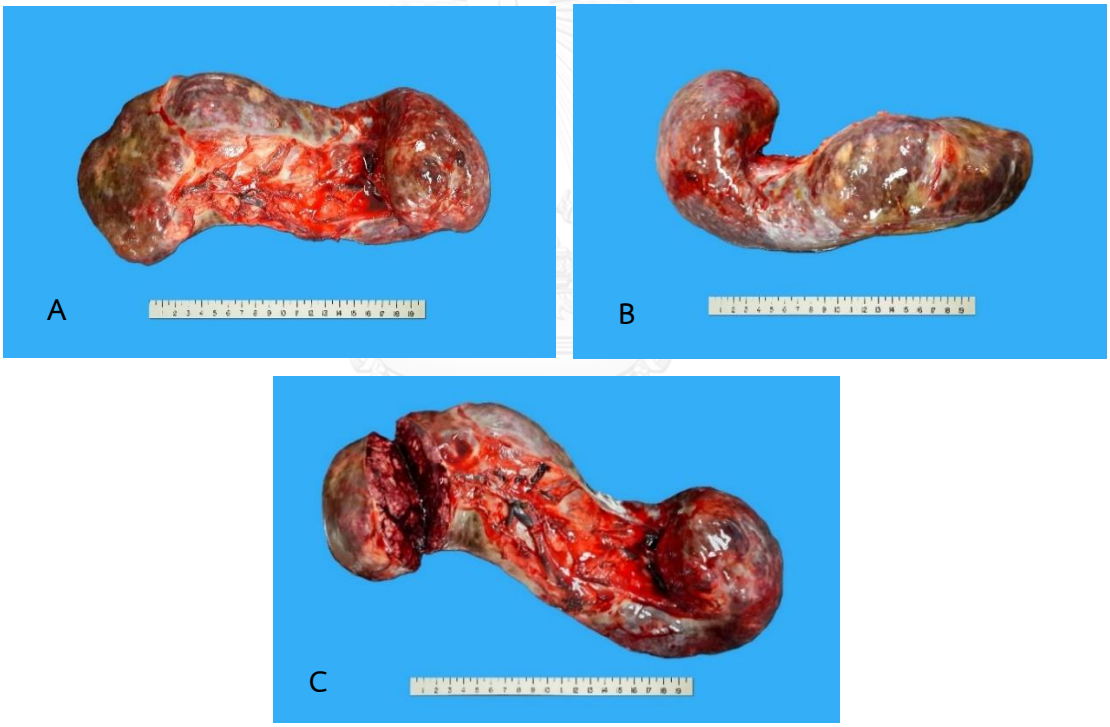


Figure 10 Canine splenic plasma cell tumor (Dog no. 30). (A) The spleen with diffusely multiple mass had smooth surface and firm consistency. (B) The lateral view of splenic tumor had dark-red with white plaque in color. (C) The cut surface of splenic mass was bulgy with white-red.

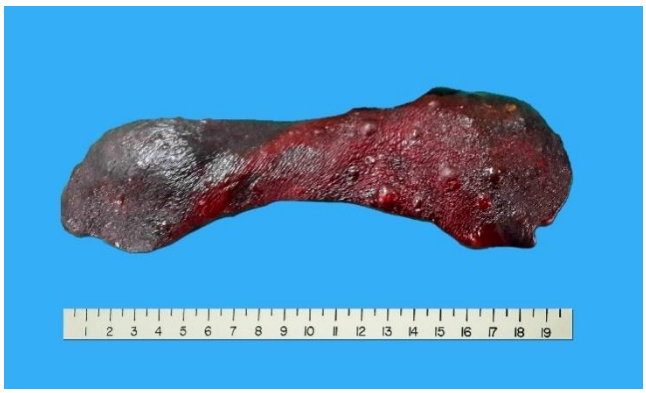


Figure 11 Mast cell tumor metastasis to spleen showed multiple masses (size 1x2 cm). There was dark-red in color and firm consistency.

Microscopic findings

After histological processes, all sections were reviewed thoroughly and several criteria were evaluated including growth pattern, blood cyst, necrosis, hemorrhage, hemosiderosis, hemophagocytosis, thrombosis, extramedullary hematopoiesis, fibrosis, mitotic figure, nuclear atypia, vascular space and amyloid deposition.

There were four growth patterns composing solid, cavernous, capillary and mixed pattern. In this study, the most common growth pattern was cavernous (46.2%, 6/13), followed by solid growth pattern (23.1%, 3/13). The capillary and mixed growth patternsequally occurred (15.4%, 2/13) (Table 4). Papillary projections with hyaline cores lined by malignant endothelial cells were shown in 4 of 13 cases (30.8%). Among papillary structures, there was 1 case (Dog no. 4) consisting of amyloid which is confirmed by Congo-Red special stain (Figure 17).

The neoplastic cells of HSA had highly heterogenous morphology. Both spindle cell-shaped and ovoid were appeared. Mitotic figures were identified in 7 out of 13 HSAs (54%). Nuclear atypia, characterized by Thomas et al. (2000), showed mild degree (23.1%, 3/13), moderate degree (13.1%, 3/13) and severe degree (53.8%, 7/13). The vascular spaces were identified as slit-like and honeycomb-like. The results showed evidence of slit-like in 1 case (8%), honeycomb-like in 9 cases (69%) and no remarkable vascular spaces in 3 cases (23%) which is assuming solid growth pattern.

Most HSA spleen showed infiltrative neoplastic cells (84.6%, 11/13) and nodular arranged tumor cells (15.4%, 2/13). The blood cyst was observed 5 out of 13 HSA (38.5%) (Figure 18). Tumors with intralesional necrosis were displayed in 8 cases (61.5%), whereas hemorrhage appeared in all cases (100%) (Figure 4-15). Most of the tumors possessed hemosiderosis (92.3%, 12/13), while hemophagocytosis and thrombosis were found in 3 cases (23.1%) and 5 cases (38.5%), respectively. Only 3 cases (23.1%) of HSA showed fibrosis and 10 cases (76.9%) displayed extramedullary hematopoiesis.

Table 4 The growth patterns of 13 splenic hemangiosarcoma in dogs

Number	Growth pattern
1	Solid (Solid 70%, cavernous 30%), (papillary projection)
2	Solid (Fibrosarcoma like spindle cell type)
3	Mixed (papillary projection)
4	Solid (papillary projection with hyaline cores), amyloid
5	Mixed
6	Cavernous (Cavernous 80%, solid 20%)
7	Cavernous
8	Capillary (Capillary60%, cavernous 40%)
9	Cavernous (Cavernous90%, solid 10%), (papillary projection)
10	Cavernous (cavernous 90%, solid 10 %)
11	Capillary (Capillary 70% solid 30%)
12	Cavernous (Cavernous 60%, solid 40%)
13	Cavernous (Cavernous 80%, solid 20%)

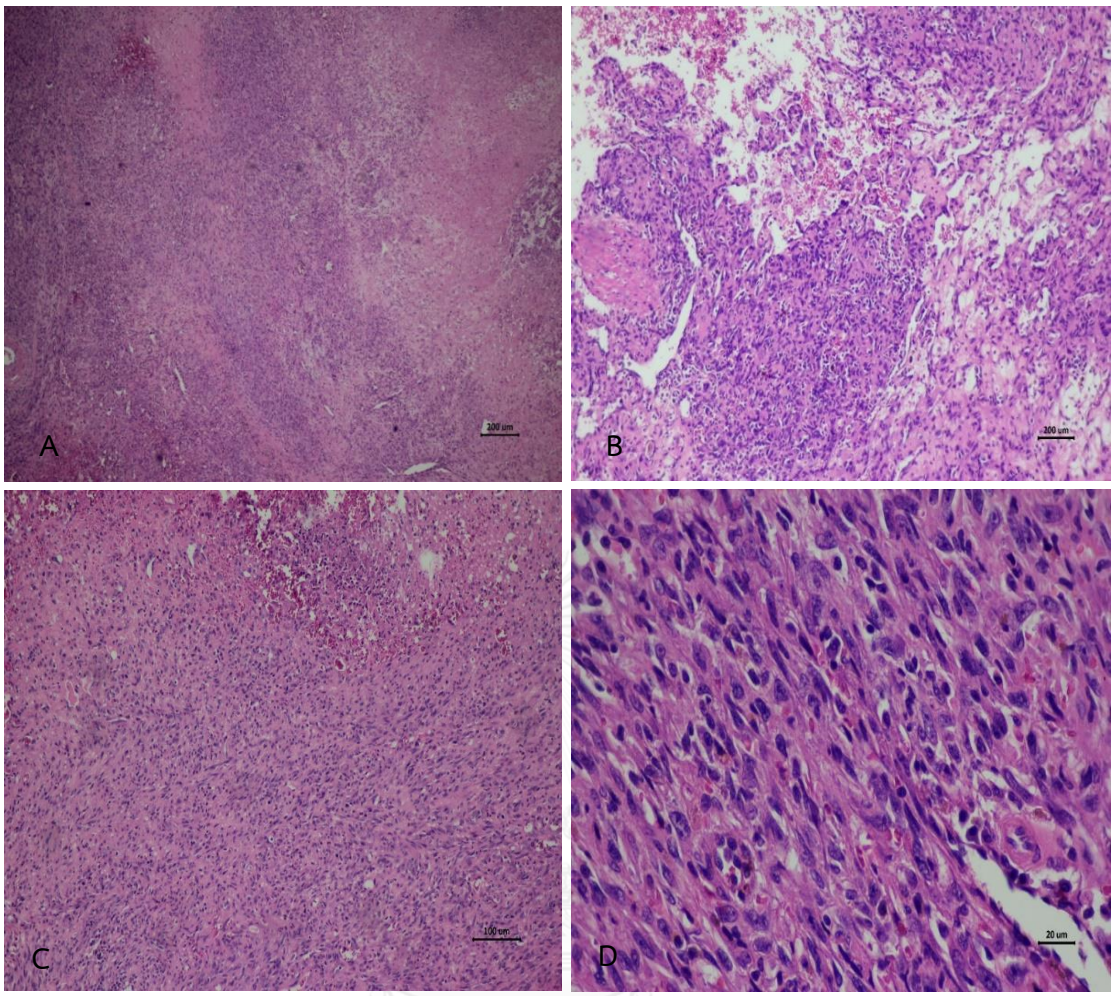


Figure 12 Solid growth pattern of canine splenic HSA. (A) Predominantly solid growth pattern. HE. Bar, 200 µm. (B) Papillary projection with hyaline cores lined by malignant cells. HE. Bar, 200 µm. (C) HSA with solid growth pattern showing spindle cells; small splits filled with erythrocytes. HE. Bar, 100 µm. (D) Solid growth pattern showing irregular vascular structures lined by malignant endothelial cells. HE. Bar, 20 µm.

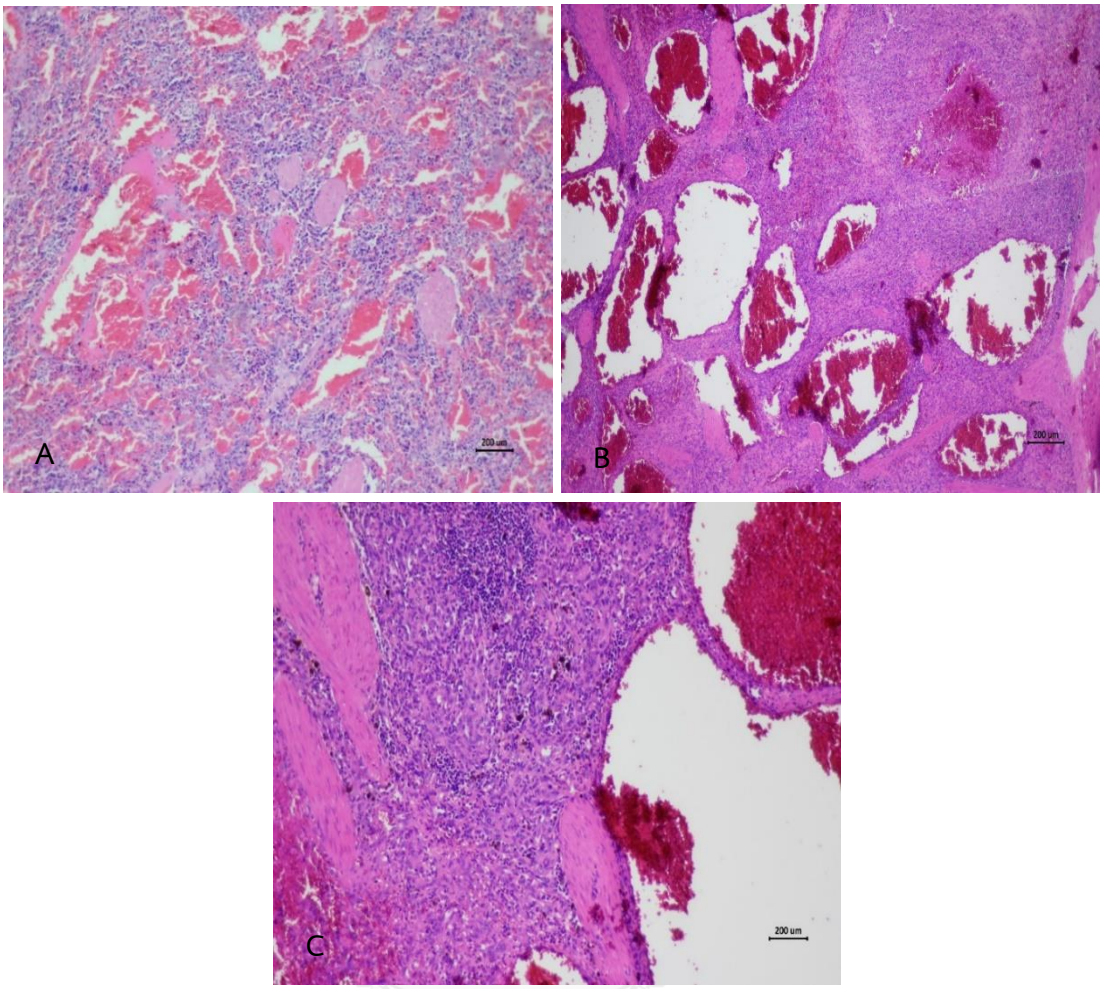


Figure 13 Cavernous growth pattern of canine splenic HSA. (A) Predominantly cavernous growth pattern; irregular structures of blood vessels filled with erythrocytes. HE. Bar, 200 µm. (B) Predominantly cavernous and partly solid growth pattern. HE. Bar, 200µm. (C) Cavernous HSA showing large blood-filled vascular space lined by endothelial cells and fascicles of spindle cells around vascular spaces. HE. Bar, 100 µm.

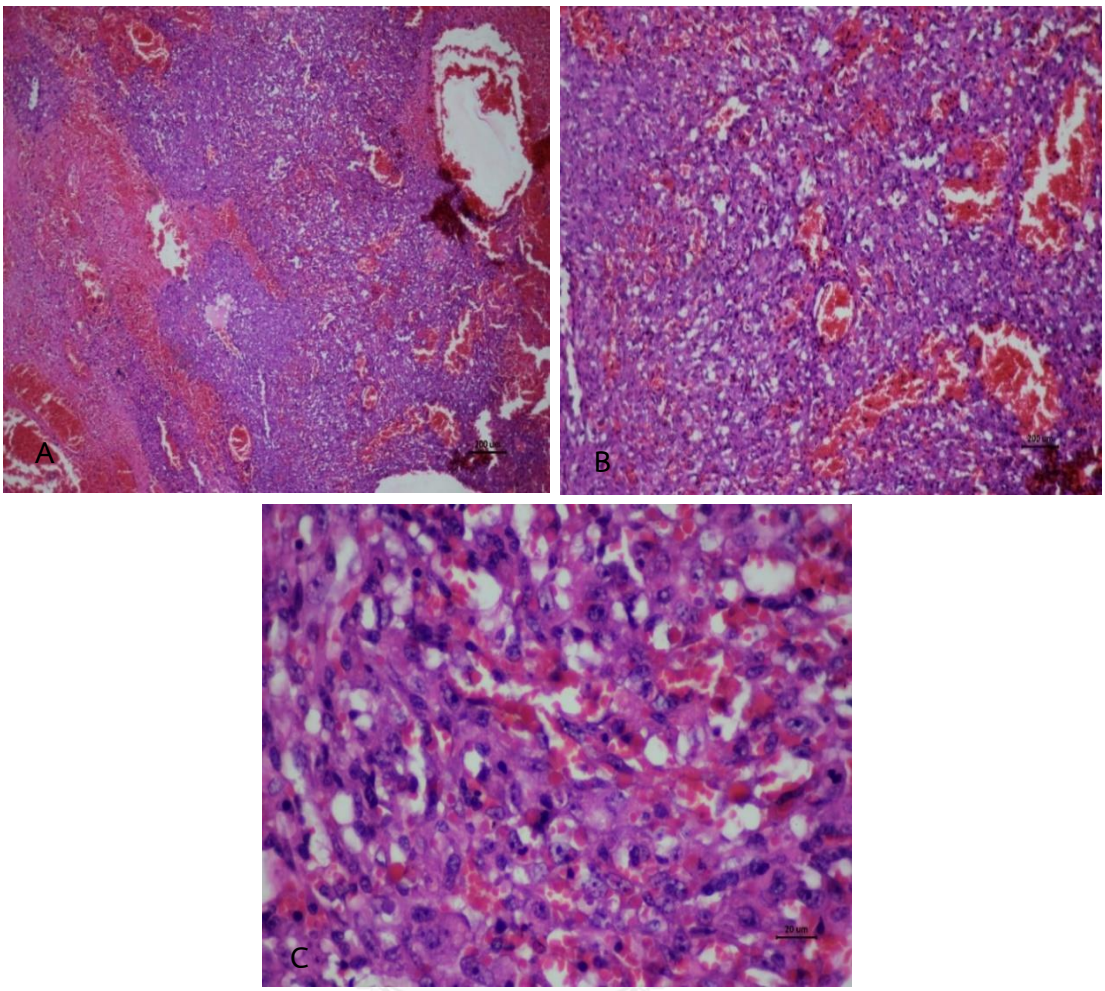


Figure 14 Capillary growth pattern of canine splenic HSA. (A) Predominantly capillary and partly cavernous growth pattern. HE. Bar, 200 µm. (B) Splenic HSA with capillary differentiation showing blood-filled vascular structures lined by malignant endothelial cells. HE. Bar, 100 µm. (C) Anastomosis vascular channels showing endothelial cells aligned on collagen trabeculae. HE. Bar, 20 µm.

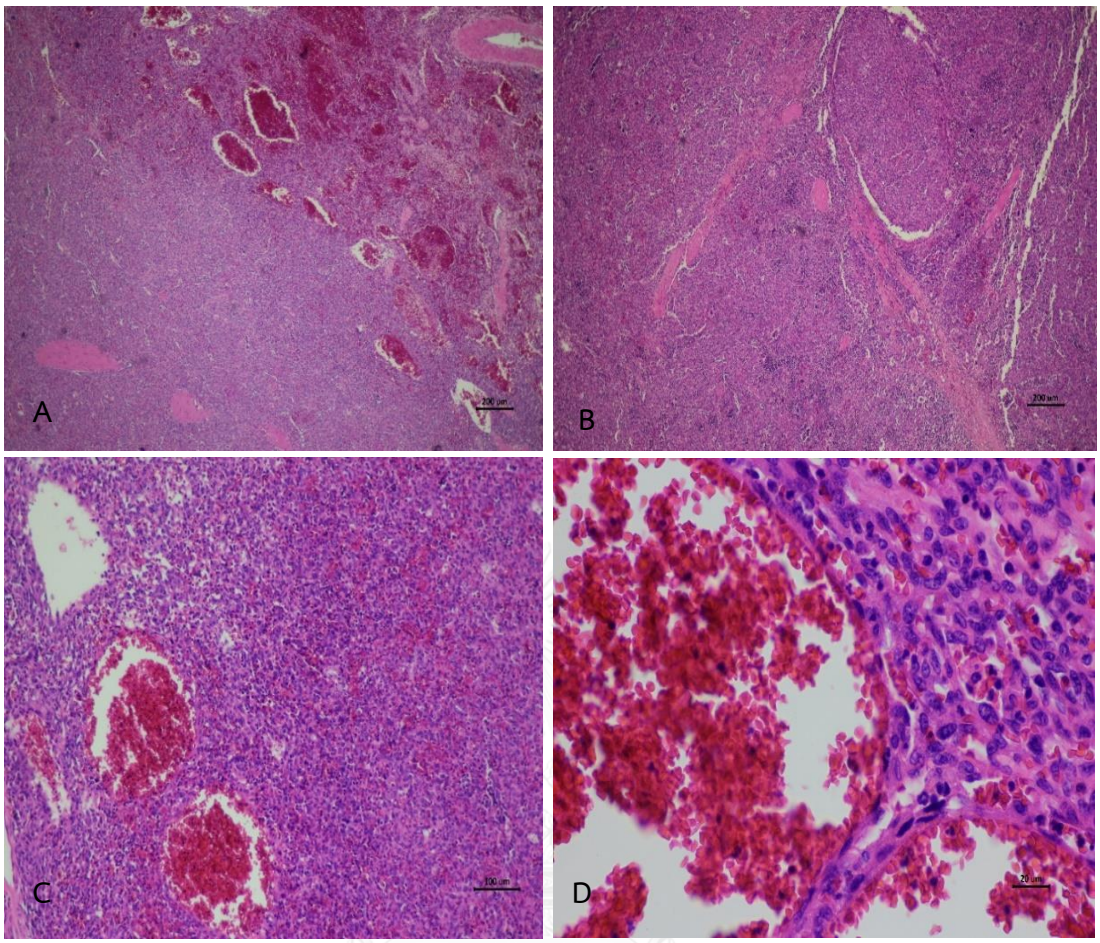
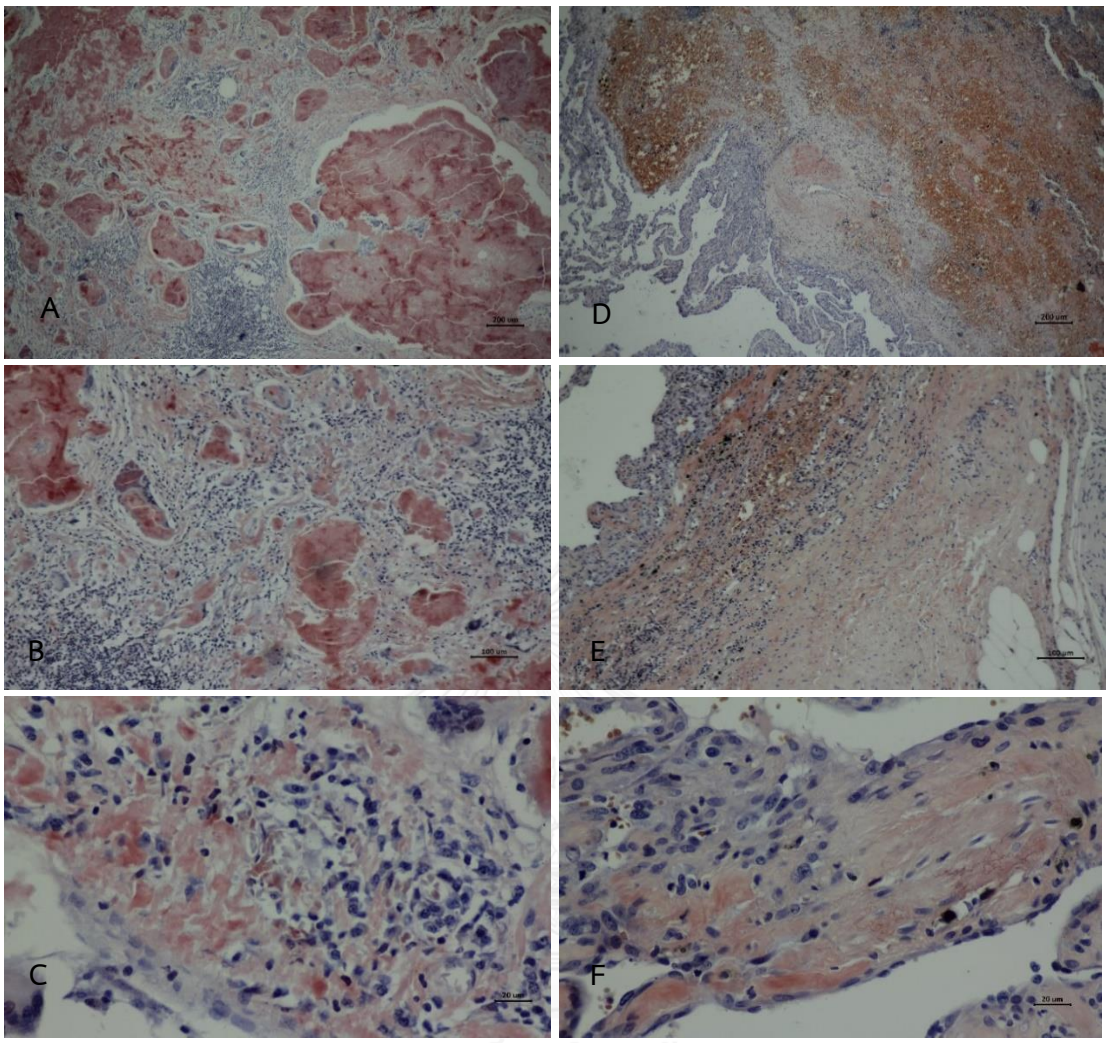
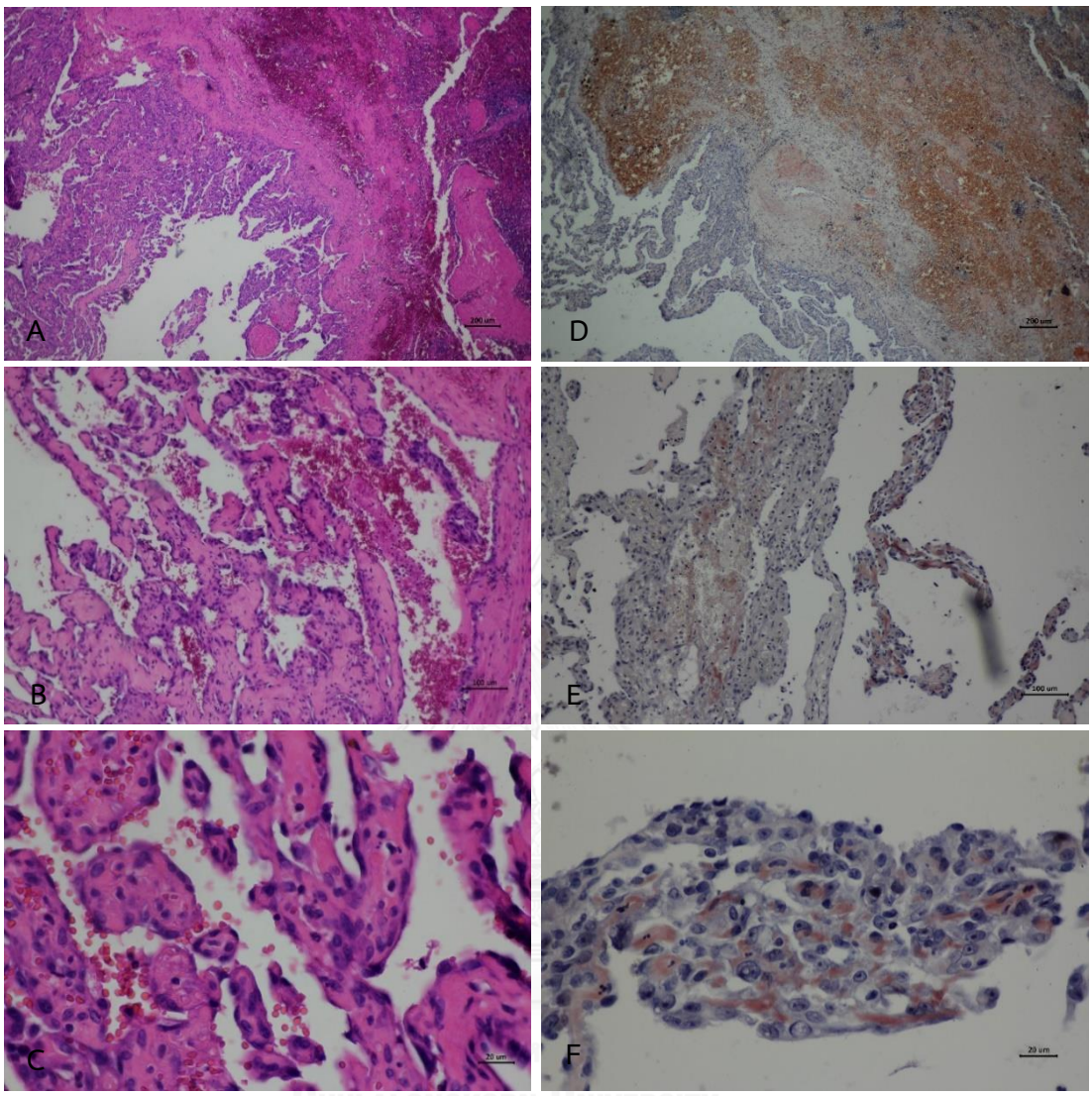


Figure 15 Mixed growth pattern of canine splenic HSA.(A) All growth patterns showing solid, cavernous and capillary growth pattern. HE. Bar, 200 µm. (B) Mixed growth pattern showing a part of solid growth pattern. HE. Bar, 200 µm. (C) Mixed growth pattern showing a part of capillary and cavernous growth pattern. HE. Bar, 100µm. (D) Blood-filled vascular space lined by malignant endothelial cells and capillary meshwork. HE. Bar, 20 µm.



จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Figure 16 Papillary. projections with hyaline cores presented amyloid locally compared with positive control. (A) Positive control showing amyloid. Congored. Bar, 200 μm. (B) Positive control showing amyloid. Congo red. Bar, 100 μm. (C) Positive control showing amyloid. Congo red. Bar, 20 μm. (D) solid growth pattern with papillary projections presented amyloid. Congo red. Bar, 200μm. (E) Papillary structures with amyloid. Congo red. Bar, 100 μm. (F) Amyloid located in hyaline cores. Congo red. Bar, 200μm (E) papillary structures with amyloid congo red. Bar, 100 μm. (F) Amyloid located in hyaline cores. Congo red. Bar, 20 μm.



CHULALONGKORN UNIVERSITY

Figure 17 Papillary projections with hyaline cores presented amyloid HE stained compared to Congo red. (A) papillary structures with hyaline cores. HE. Bar, 200 μm. (B) Solid growth pattern with amyloid deposition. HE. Bar, 100 μm. (C) Hyaline cores lined by malignant endothelial cell. HE. Bar, 20 μm. (D) solid growth pattern with papillary projections presented amyloid. Congo red. Bar, 200 μm. (E) Amyloid. Congo red. Bar, 100 μm. (F) Amyloid located in hyaline cores. Congo red. Bar, 20 μm.

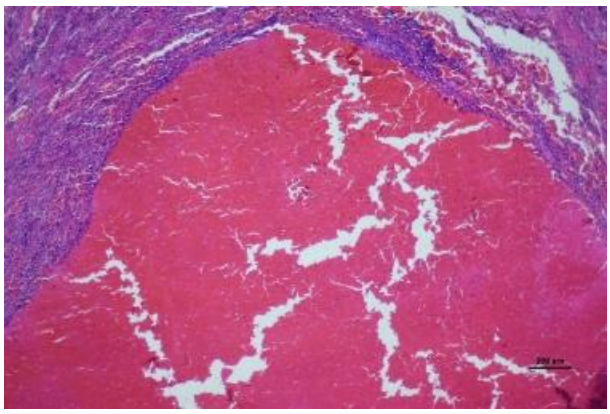


Figure 18 Blood cyst in canine splenic HSA. HE. Bar, 200 µm.

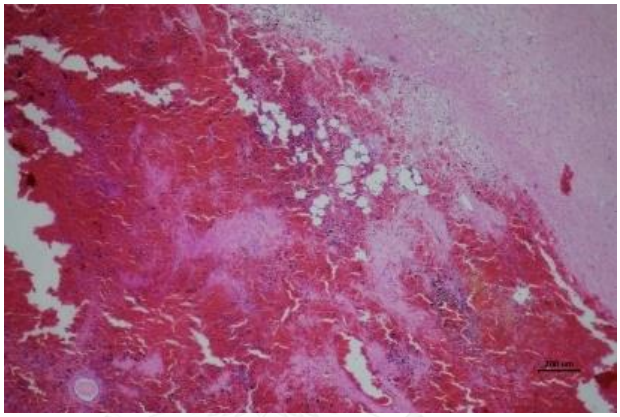


Figure 19 Hemorrhage in canine splenic HSA. HE. Bar, 200 µm.

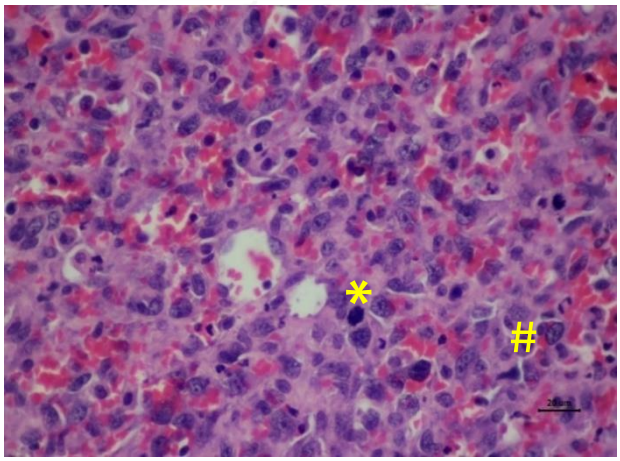


Figure 20 Hemophagocytosis (#), mitotic figures (*) and nuclear atypia in canine splenic HSA. HE. Bar, 20 µm.

D. Canine serum Ang-2 concentration in OVH-operated healthy dogs and dogs with splenic lesions

There was significant difference between control group and splenic mass group (P=0.03). The mean Ang-2 of control group was 1,583 pg/ml (range 500-3,250 pg/ml, SD=1,085), while median was 1,050 pg/ml. The splenic mass group had mean Ang-2 at 3,142 pg/ml (range 600-11,800 pg/ml, SD=2,767), whereas median was 1,781 pg/ml. (Figure 21)

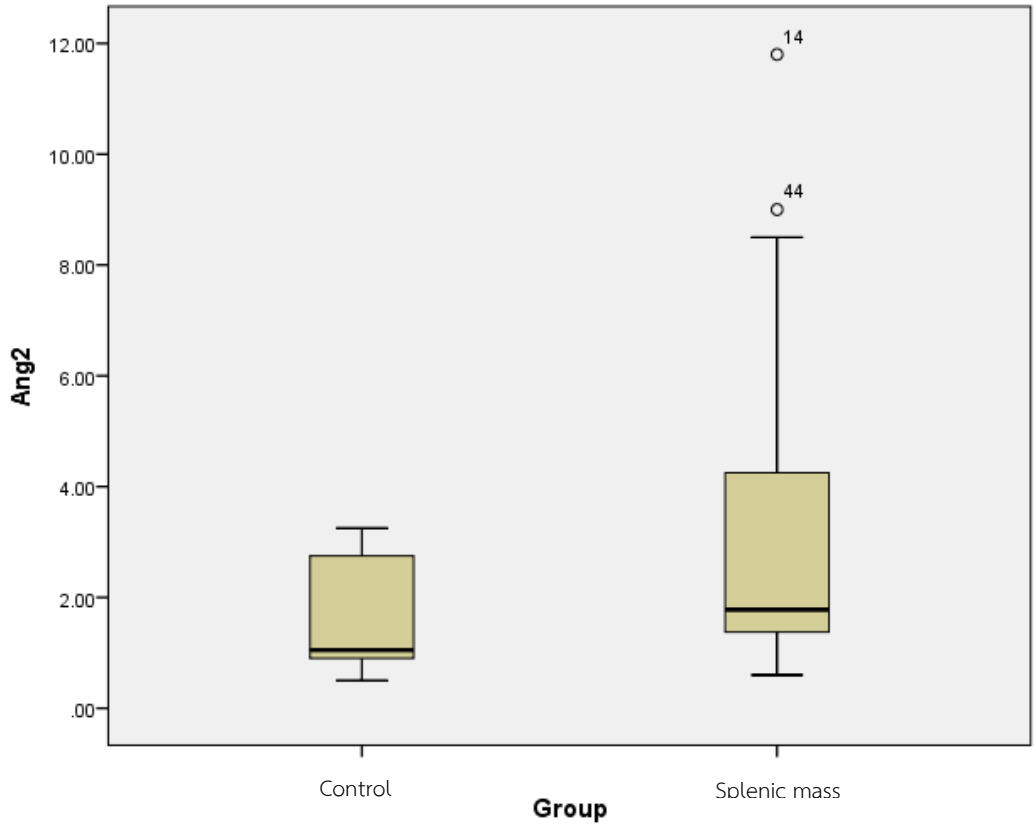


Figure 21 Box plot displayed control group (Mean 1,583, SD=1,085, median 1,050) and splenic mass tumor (Mean 3,142, SD=2,767, median 1,781).

There was no significant difference between HSA group and splenic mass group (P=0.132). The mean Ang-2 of HSA group was 1,681 pg/ml (range 1,100-3,000 pg/ml, SD=631), while median was 1,450 pg/ml. The splenic mass group had mean Ang-2 at 3,142 pg/ml (range 600-11,800 pg/ml, SD=2,767), whereas median was 1,781 pg/ml. (Figure 22)

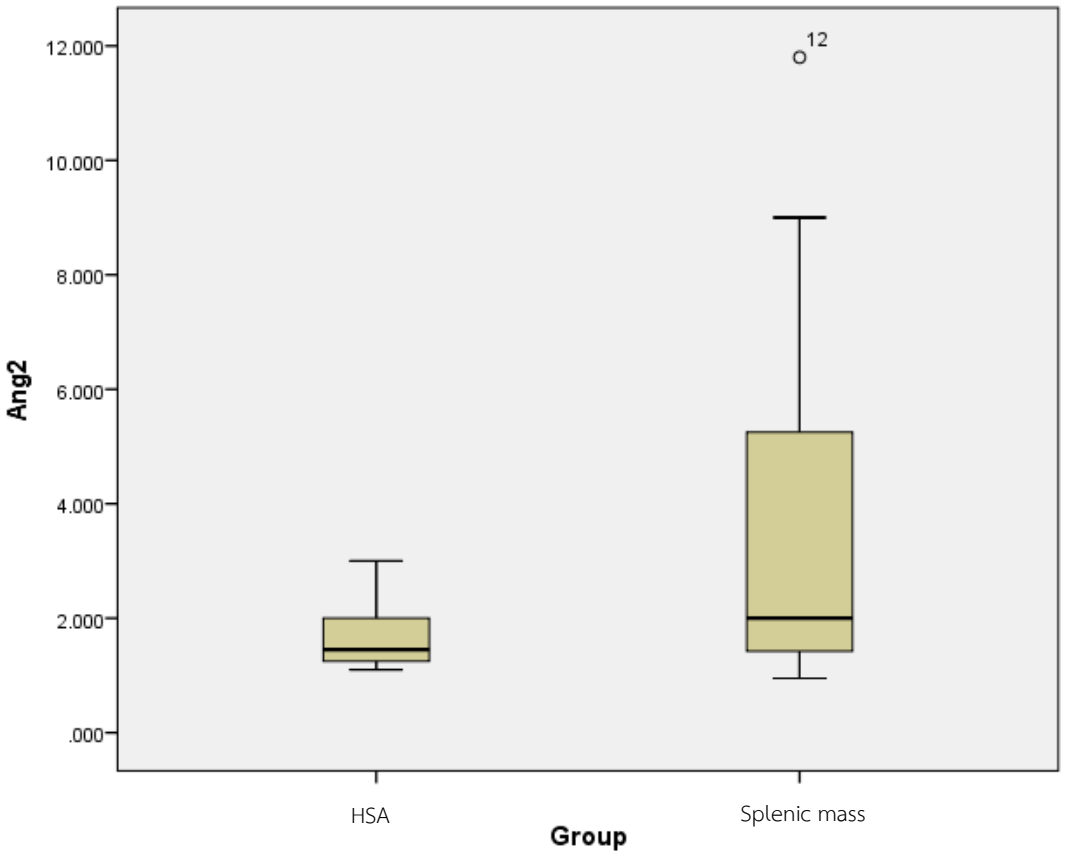


Figure 22 Box plot displayed HSA group (Mean 1,681, SD=631, median 1,450) and splenic mass tumor (Mean 3,142, SD=2,767, median 1,781).

There was no significant difference between control group, splenic tumor group and non-splenic tumor group (P=0.194). The mean Ang-2 of control group was 1,583 pg/ml (range 500-3,250 pg/ml, SD=1,085), while median was 1.050 pg/ml. The splenic tumor group had mean Ang-2 at 2,837 pg/ml (range 600-11,800 pg/ml, SD=2,659), whereas median was 1,562 pg/ml. The mean Ang-2 of non-splenic tumor group was 3,850 pg/ml (range 950-9,000 pg/ml, SD=3.000) and median was 3,125 pg/ml (Figure 23).

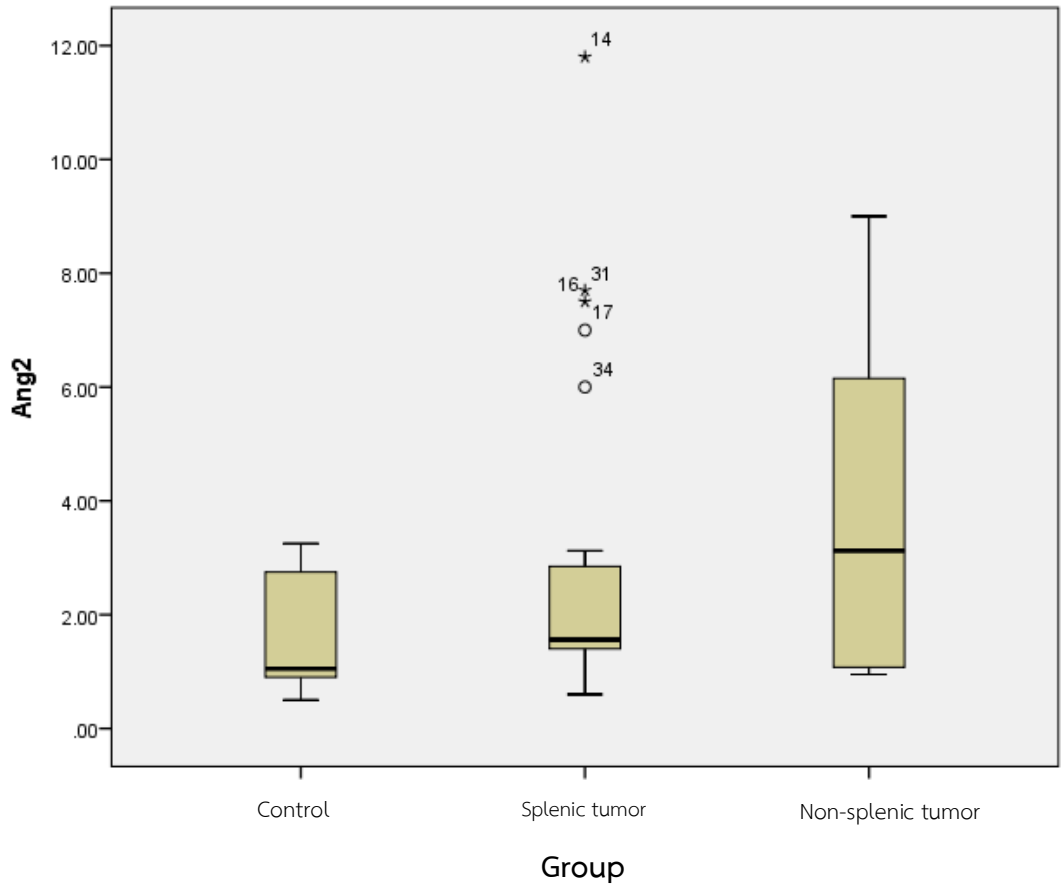


Figure 23 Box plot displayed control group (Mean 1,583, SD=1,085, median 1,050), splenic tumor group (Mean 2,837, SD=2,659, median 1,562) and non-splenic tumor (Mean 3,850, SD=3,000, median 3,125).

There was no significant difference between splenic malignant tumor and benign tumor group (P=0.094). The mean of the malignant tumor group was 2,174 pg/ml (range 600-7,700 pg/ml, SD=1,695) and the median was 1,560 pg/ml, while the mean of benign tumor group was 4,829 pg/ml (range 1,350-11,800 pg/ml, SD=4,010) and the median was 2,700 pg/ml (Figure 24).

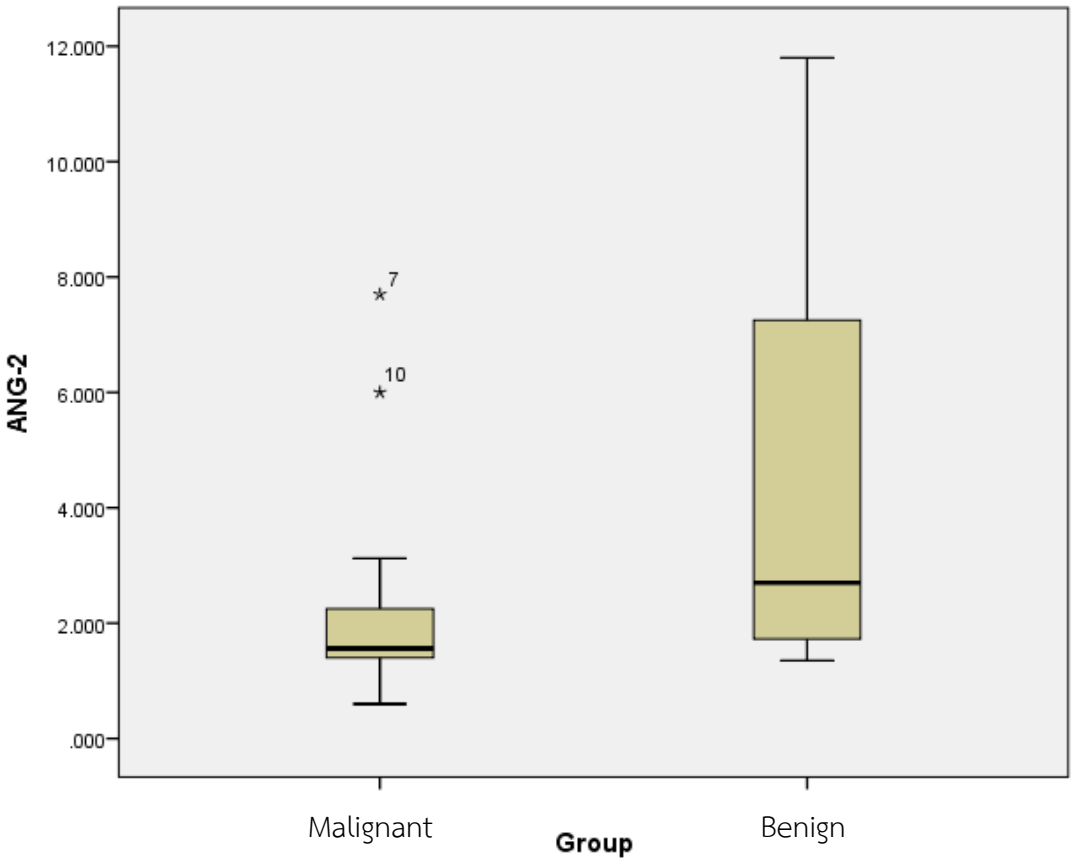


Figure 24 Box plot showed serum Ang-2 level in malignant tumor group (Mean 2,174, SD=1,695, median 1,560) and in benign tumor group (Mean 4,829, SD=4,010, median 2,700).

However, dogs with splenic HSA had significantly lower serum Ang-2 concentrations compared to dogs with hemangioma (P=0.019), but not different from healthy dogs (P=0.204). Hemangioma group had significantly higher serum Ang-2 concentrations compared to normal group (P=0.034). The mean Ang-2 level of splenic HSA group was 1,681 pg/ml (range 1,100-3,000 pg/ml, SD=631), while the median is 1,450 pg/ml, whereas the mean Ang-2 in splenic hemangioma was 5,408 pg/ml (range 1,450-11,800 pg/ml, SD=4,059) and the median was 4,850 pg/ml (Figure 25). Furthermore, Ang-2 concentrations displayed no significant difference between control group (P=0.074), HSA (P=0.500) and hemangioma (P=0.593) either before or after surgery.

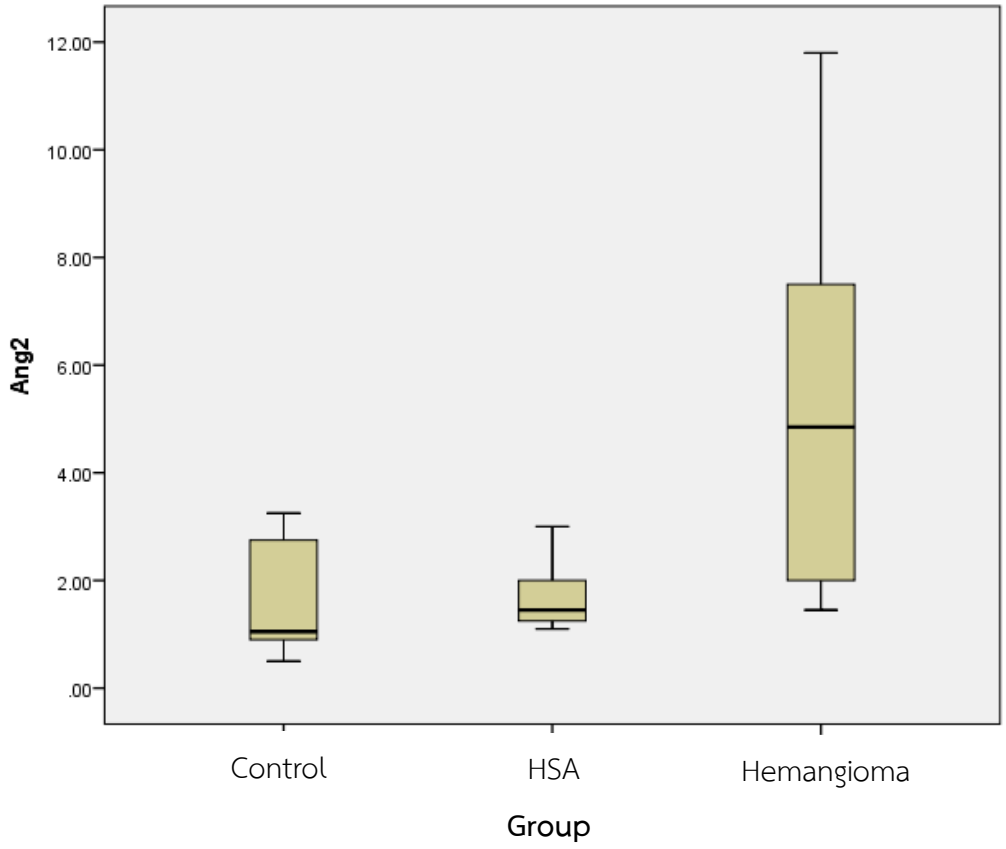


Figure 25 Box plot displayed control group (Mean 1,583, SD=1,085, median 1,050) splenic HSA group (Mean 1,681, SD=631, median 1,450) and hemangioma group (Mean 5,408, SD=4,059, median 4,850).

Serum Ang-2 concentration in dogs with splenic HSA showed no significant correlation between clinical staging (Figure 26) (r=-0.247). However, a few dogs were classified in clinical stage 1 (n=1) and 2 (n=2) resulting in undetermined results. The largest group consisted of HSA dogs with clinical stage 3 (n=8). They expressed the serum Ang-2 concentrations were documented as followed: stage 1 (1,400 pg/ml), stage 2 (3,000 pg/ml), stage 3 (1,250 pg/ml, 2,500 pg/ml, 1,500 pg/ml, 1,100 pg/ml, 1,560 pg/ml, 2,000 pg/ml, 1,400 pg/ml and 1,100 pg/ml).

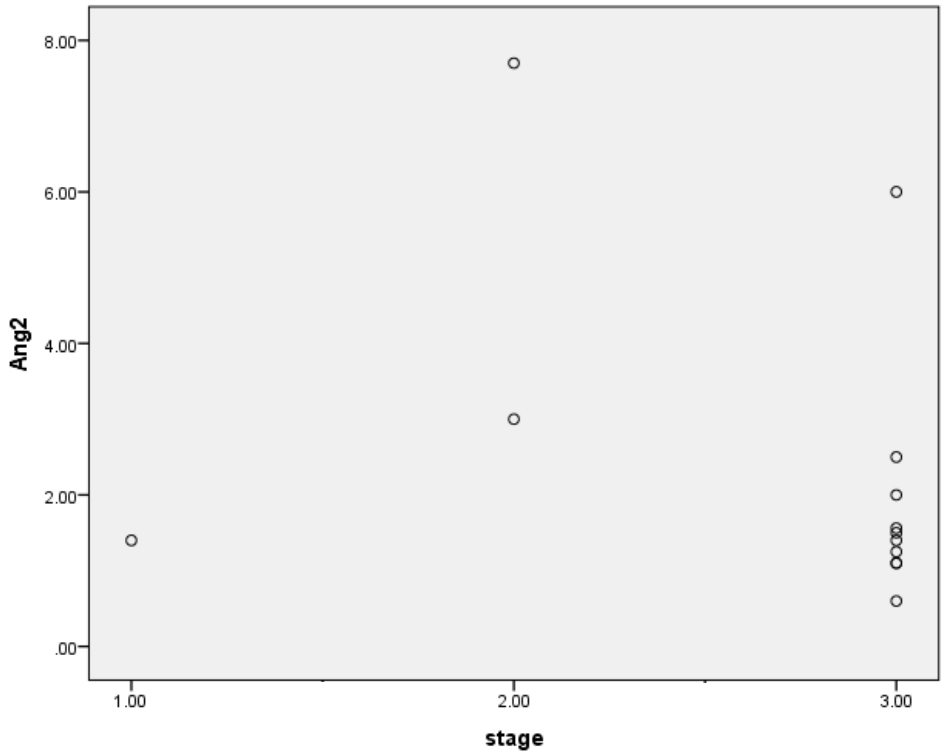


Figure 26 Serum Ang-2 concentration of the dogs with splenic HSA in different stages.

E. Survival time of dogs with HSA

The date of surgery and the date of death were recorded in all cases (except dog no. 6) (table 2). The latest survival time was recorded on February 2016. The mean survival time was 80.57 days (SD=25.88), while the median survival time was 56 days (range 0 – 264 days) (Figure 27). The shortest survival time was found in splenic HSA with rupture. Furthermore, the longest survival time was found in HSA dog with stage 2 (Dog no. 8).

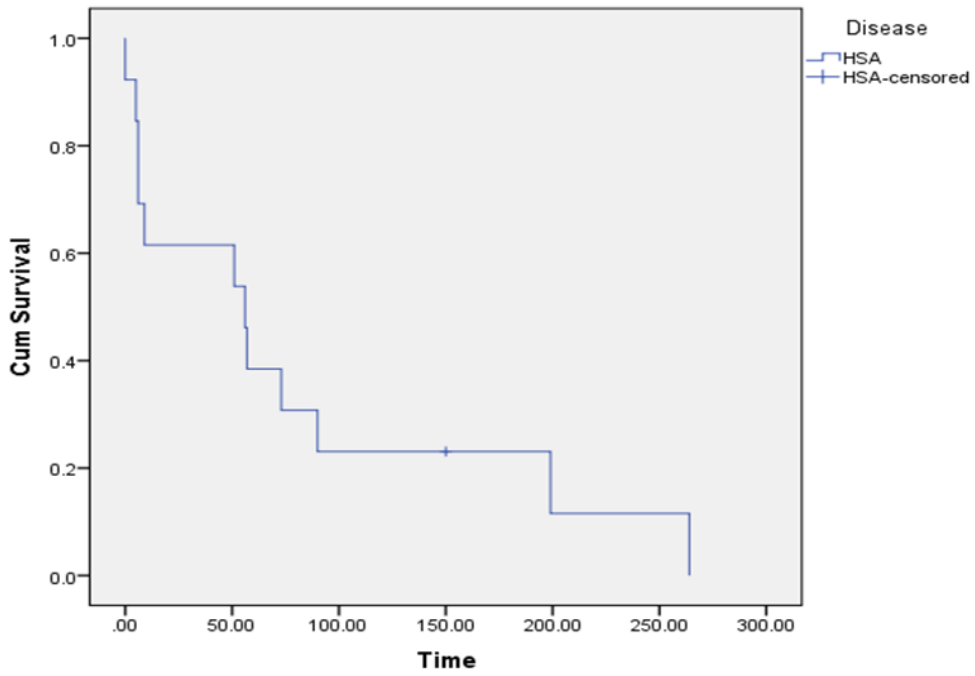


Figure 27 Kaplan-Meier survival plot of 13 dogs with HSA. The mean survival time is 80 days, (median 56 days; range 0 – 264 days).

All growth pattern of splenic HSA had no significant difference in survival time (Figure 28). The dogs with solid growth pattern of splenic HSA had a median survival time for 56 days. While the median survival time in dogs with cavernous growth pattern was 9 days, in dogs with capillary growth pattern was 6 days, and in dogs with mixed pattern was 0 days. The mean survival time of solid growth pattern was 102 days (range 51-199 days, SD=48.52), meanwhile the mean survival time of cavernous, capillary, and mixed growth pattern were 55 days (range 5-90 days, SD=22.10), 135 days (range 6-264 days, SD=129), and 28 days (range 0-57 days, SD=28.50) respectively. In this study, there was one dog having died during splenectomy (Dog no. 5). Thus the shortest survival time was found in mixed growth pattern.

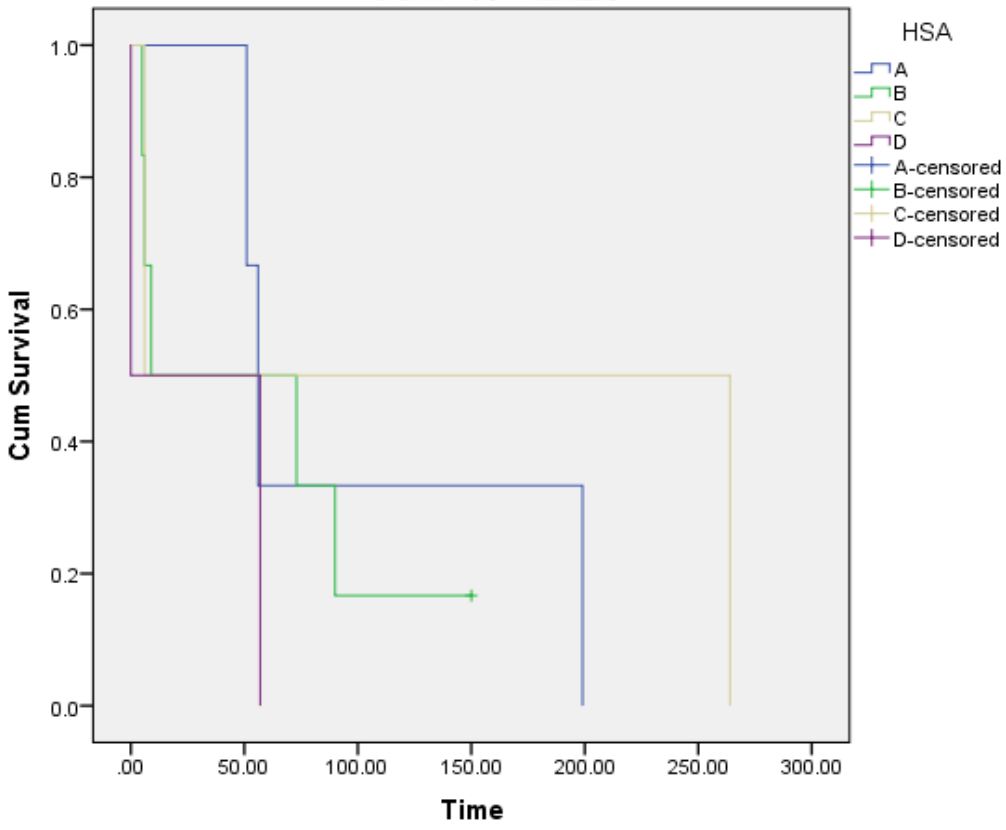


Figure 28 Kaplan-Meier survival plot of 13 dogs with splenic HSA of various growth patterns. (A) Solid growth pattern, (B) Cavernous growth pattern, (C) Capillary growth pattern, (D) Mixed growth pattern

CHAPTER V
DISCUSSION AND CONCLUSION

Discussion

The general characteristic of the dogs with splenic masses revealed that large breed dogs were risk of developing the splenic lesions comparing to smaller breed. Splenic HSA were found mainly in large breed dogs such as Labrador and Golden retriever. The old-aged dogs were prone to develop splenic diseases including splenic HSA. It was in agreement with a previous study indicated that old-aged and large breed are predisposing factors for HSA (Christensen et al., 2009). Besides, similar to an other's report, males were overrepresented especially neutered male (Sabattini and Bettini, 2009)

As reported by Gamlem et al. (2008), the most common splenic tumor is HSA. The histopathological results of canine splenic abnormalities were varied including non-tumor, benign and malignant tumors (Gamlem and Nordstoga, 2008). Our result revealed that approximately one third of dogs with splenic mass and two third of malignant cancer of the spleen were HSA. The gross lesions of splenic abnormalities, including tumor size, location of mass and characteristic of tumor, were not specific for HSA. While the histological features are able to identify HSA. Thomas et al. (2000) recommends the use of other parameters to distinguish the malignant from the benign tumors; for examples, hemophagocytosis, extramedullary hematopoiesis, vascular space, necrosis, mitotic figures, and nuclear atypia. The findings of a previous study were that primary splenic angiosarcoma in human has papillary structures with hyaline cores and focal amyloid deposit (Neuhauser et al., 2000). This study also found papillary structures with amyloid deposition in canine splenic HSA.

The microscopic features were used to distinguish the growth pattern of canine splenic HSA. The most common growth pattern was cavernous, followed by solid growth pattern. While the capillary growth pattern and mixed growth pattern occurred equally. In contrast, Goritz et al. (2013) reported that a mixed pattern of cavernous, capillary and solid neoplasm was mostly found in canine splenic HSA. This might

indicate that HSA has various growth patterns. Therefore, sample collection from several sections of the tumors should be done.

In 2013, Goritz et al. revealed that the median survival time was short duration after splenectomy. Additionally, growth patterns had no significant difference in survival time, similar to the finding of this study. However, our study found that the shortest survival time was shown in the dog with mixed growth pattern, while it was found in the dog with cavernous growth pattern in another study (Goritz et al., 2013). The cause of death in this case may be from a rupture of large splenic mass leading to hypovolemic shock. The stronger point of this view is that the tumor size and rupture affect the survival time. Therefore, the growth pattern may not relate with the survival time.

Stage of splenic HSA are not related to the survival time (Wood et al., 1998). It is supported by our study. Any stages of the HSA dogs with splenic mass rupture is risk to have a shorter survival time than that without rupture became of internal bleeding. Moreover, metastatic mass of splenic HSA may also cause shorter survival time after splenectomy.

A previous study revealed that Ang-2 marker showed higher expression in human cutaneous angiosarcomas comparing with normal tissue (Brown et al., 2000). Accordingly, Goritz et al. (2013) demonstrated that Ang-2 may be useful as an immunohistochemical marker for canine neoplastic endothelial cell in splenic HSA. Therefore, this study investigated a correlation between serum Ang-2 concentration in canine splenic HSA comparing with healthy dogs by ELISA determination. The results revealed that there was no significant difference of Ang-2 concentration between splenic HSA and healthy dogs. While the Ang-2 concentration was higher in dogs with splenic hemangioma in comparison with healthy and HSA dogs. The evidence of decreased serum Ang-2 concentration could be explained by the impairment of Ang-Tie system. Although the vasculogenesis remains at tumor margin during tumor growth, it seems to be decreased because of the evidence of central intralesional necrosis and loss of massive tumor cells. Ang-2 plays an important role in angiogenesis and vascular remodeling. It may be down-regulated in splenic HSA. In human angiosarcoma, there are also reduced expression of Ang-2. It is relative to non-vasoformative cancer which

is clinically progressive tumor (Buehler et al., 2013). In addition, Ang-Tie system may function in a paracrine and autocrine (Shim et al., 2007). The activated endothelial cell releases Ang-2 from Weibel-palade bodies to bind Tie-2 receptor on endothelial cells. The activation of Ang-Tie system was occurred directly on endothelial cells. It is possible that the Ang-2 concentration may not increase in blood stream. Thus, Ang-2 expression was not a robust serum marker for canine splenic HSA. While it may be helpful to be a tissue marker for canine neoplastic tumors (Goritz et al., 2013). A small sample size might also affect the statistical analysis.

In contrast, Ang-2 concentration was elevated in several studies. For example, Ang-2 concentration in ovarian cancer patients was higher than in healthy women (Sallinen et al., 2010). Fawzy et al. (2012) reported that serum Ang-2 concentration was increased in human with lung cancer compared with healthy ones. Engin et al. (2012) showed an elevated Ang-2 concentration in colon cancer (Engin et al., 2012). Similarly, Wang et al. (2015) reported that serum Ang-2 level in patients with colorectal cancer was higher than that of healthy people (Wang et al., 2015). The elevation of Ang-2 concentration correlates with some cancers in human such as breast cancer (Sfiligoi et al., 2003) and melanoma (Helfrich et al., 2009). Not only has the previous study presented elevation of Ang-2 concentration in various cancers, this study also presented it in splenic mass. However, our observations found that serum Ang-2 level tended to elevate in the HSA dogs with metastatic melanoma and with metastatic sertoli cell tumor. These findings may associate with neovascular formation in metastatic tumors. As a result, the trend is toward a higher Ang-2 concentration in splenitis. It may be caused from increased Ang-2 expression during inflammatory process of spleen (Fiedler et al., 2004).

For splenic hemangioma, the elevation of serum Ang-2 level may associate with local hypoxia. It leads to proliferation of endothelial cells and tumor angiogenesis (Cao et al., 2007). Ang-2 is significantly expressed in endothelial cells and it will increase the expression when angiogenesis was occurred (Moreira et al., 2007). Furthermore, serum Ang-2 level was elevated in splenic hemangioma dogs compared with HSA and healthy dogs. As reported by Willcox et al., (2000), splenic hemangioma with a large mass or multiple masses were risk to a malignant transformation into splenic

angiosarcoma in human. Similar to another's report, there was also malignant transformation of human cutaneous hemangioma into angiosarcoma (Nathenson et al., 2014). Even transformation of benign hemangioma into malignant angiosarcoma was rarely. However, there was several reports of malignant transformation in human (Willcox et al., 2000; Jeng et al., 2014; Nathenson et al., 2014). The possibility that the malignant transformation of splenic hemangioma into HSA may be occurred in dogs. Nevertheless, there was no report in dog presenting malignant transformation of splenic hemangioma. Therefore, the elevation of serum Ang-2 concentration may be useful as a serum marker for canine splenic hemangioma. In addition, serum Ang-2 level may be used for monitoring splenic hemangioma before malignant transformation.

In present study, there are no significant difference between splenic malignant tumor and benign tumor and between splenic mass and non-mass. All splenic abnormalities may lead to inflammation and angiogenesis. As Ang-2 has an influence on physiologic and pathogenic vessel sprouting, therefore, the expression of Ang-2 concentration is increased. Also found, serum Ang-2 levels are not significantly different between before and after surgery. It may be caused from collecting blood sample after surgery for only 10 days. This period is relative short in human's study. Nevertheless there is no previous report in dogs. As reported by Kopczyńska et al. (2012), serum Ang-2 level of human with lung cancers before surgery was approximate to that at 7 days after surgery, then, Ang-2 concentration was reduced until 30 days after surgery.

Investigating HSA, the correlation between stages of HSA and Ang-2 concentrations were not significant. Accordingly, Engin et al. (2012) reported that there were weak correlation between stages of HSA and Ang-2 level. For further study, a larger sample size should be used to investigate the role of Ang-2 in canine splenic HSA.

Conclusion

Splenic HSA is frequently observed in old-aged dogs. Golden and Labrador retrievers are predisposing breeds for HSA. It also noted that males were affected more than females. According to biopsy, HSA is the most common splenic tumors. A shorter survival time may be related to tumor size and rupture. Additionally, the survival time of canine splenic HSA is not associated with growth pattern and stage of the tumor. Serum Ang-2 level of splenic HSA does not differ from other splenic abnormalities, whereas Ang-2 concentration of splenic hemangioma dogs is higher than HSA and healthy dogs. Therefore, evaluation of serum Ang-2 concentration may not be helpful in the diagnosis of canine splenic HSA. Although, the increase of serum Ang-2 concentration may be found in splenic hemangioma. Serum Ang-2 level may be used for monitoring splenic hemangioma before malignant transformation. In addition, there was no correlation between Ang-2 concentrations and stages of HSA. A larger sample size should be used in further studies. Moreover, evaluation of the expression of Ang-1 and Tie receptor should be investigated in order to understand the role of Ang-Tie system and to find the tumor marker for canine splenic HSA.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

REFERENCES

Bertazzolo W, Dell'Orco M, Bonfanti U, Ghisleni G, Caniatti M, Masserdotti C, Antoniazzi E, Crippa L and Roccabianca P 2005. Canine angiosarcoma: cytologic, histologic, and immunohistochemical correlations. Vet Clin Pathol. 34(1): 28-34.

Brown LF, Dezube BJ, Tognazzi K, Dvorak HF and Yancopoulos GD 2000. Expression of Tie1, Tie2, and angiopoietins 1, 2, and 4 in Kaposi's sarcoma and cutaneous angiosarcoma. Am J Pathol. 156(6): 2179-2183.

Brown NO, Patnaik AK and MacEwen EG 1985. Canine hemangiosarcoma: retrospective analysis of 104 cases. J Am Vet Med Assoc. 186(1): 56-58.

Buehler D, Rush P, Hasenstein JR, Rice SR, Hafez GR, Longley BJ and Kozak KR 2013. Expression of angiopoietin-TIE system components in angiosarcoma. Mod Pathol. 26(8): 1032-1040.

Cao Y, Sonveaux P, Liu S, Zhao Y, Mi J, Clary BM, Li C-Y, Kontos CD and Dewhirst MW 2007. Systemic Overexpression of Angiopoietin-2 Promotes Tumor Microvessel Regression and Inhibits Angiogenesis and Tumor Growth. Cancer Research. 67(8): 3835-3844.

Chikazawa S, Hori Y, Hoshi F, Kanai K, Ito N and Higuchi S 2013. Hyperferritinemia in dogs with splenic hemangiosarcoma. J Vet Med Sci. 75(11): 1515-1518.

Christensen N, Canfield P, Martin P, Krockenberger M, Spielman D and Bosward K 2009. Cytopathological and histopathological diagnosis of canine splenic disorders. Aust Vet J. 87(5): 175-181.

Clifford CA, Hughes D, Beal MW, Mackin AJ, Henry CJ, Shofer FS and Sorenmo KU 2001. Plasma vascular endothelial growth factor concentrations in healthy dogs and dogs with hemangiosarcoma. J Vet Intern Med. 15(2): 131-135.

Clifford CA, Mackin AJ and Henry CJ 2000. Treatment of Canine Hemangiosarcoma: 2000 and Beyond. Journal of Veterinary Internal Medicine. 14(5): 479-485.

Davis S, Papadopoulos N, Aldrich TH, Maisonpierre PC, Huang T, Kovac L, Xu A, Leidich R, Radziejewska E, Rafique A, Goldberg J, Jain V, Bailey K, Karow M, Fandl J, Samuelsson SJ, Ioffe E, Rudge JS, Daly TJ, Radziejewski C and Yancopoulos GD 2003. Angiopoietins have distinct modular domains essential for receptor binding, dimerization and superclustering. Nat Struct Biol. 10(1): 38-44.

Dumont DJ, Gradwohl G, Fong GH, Puri MC, Gertsenstein M, Auerbach A and Breitman ML 1994. Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. Genes Dev. 8(16): 1897-1909.

Eberle N, von Babo V, Nolte I, Baumgärtner W and Betz D 2012. Umfangsvermehrungen der Milz beim Hund. Teil 1: Epidemiologische Aspekte, klinische Charakteristika sowie histopathologische Befunde von 249 Fällen (2000–2011). Tierärztliche Praxis Kleintiere. 40(4): 250-260.

Eilken HM and Adams RH 2010. Dynamics of endothelial cell behavior in sprouting angiogenesis. Curr Opin Cell Biol. 22(5): 617-625.

Engin H, Ustundag Y, Tekin IO, Gokmen A, Ertop S and Ilikhan SU 2012. Plasma concentrations of angiopoietin-1, angiopoietin-2 and Tie-2 in colon cancer. Eur Cytokine Netw. 23(2): 68-71.

Etoh T, Inoue H, Tanaka S, Barnard GF, Kitano S and Mori M 2001. Angiopoietin-2 Is Related to Tumor Angiogenesis in Gastric Carcinoma. Possible in Vivo Regulation via Induction of Proteases . 61(5): 2145-2153.

Fagiani E and Christofori G 2013. Angiopoietins in angiogenesis. Cancer Lett. 328(1): 18-26.

Ferrer L, Fondevila D, Rabanal RM and Vilafranca M 1995. Immunohistochemical detection of CD31 antigen in normal and neoplastic canine endothelial cells. J Comp Pathol. 112(4): 319-326.

Fiedler U and Augustin HG 2006. Angiopoietins: a link between angiogenesis and inflammation. Trends Immunol. 27(12): 552-558.

Fiedler U, Scharpfenecker M, Koidl S, Hegen A, Grunow V, Schmidt JM, Kriz W, Thurston G and Augustin HG 2004. The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies. Blood. 103(11): 4150-4156.

Folkman J 1995. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med. 1(1): 27-31.

Frenz M, Kaup FJ and Neumann S 2014. Serum vascular endothelial growth factor in dogs with haemangiosarcoma and haematoma. Res Vet Sci. 97(2): 257-262.

Fukumoto S, Miyasho T, Hanazono K, Saida K, Kadosawa T, Iwano H and Uchide T 2015. Big endothelin-1 as a tumour marker for canine haemangiosarcoma. Vet J. 204(3): 269-274.

Furuya M, Yonemitsu Y and Aoki I 2009. III. Angiogenesis: Complexity of Tumor Vasculature and Microenvironment. Current Pharmaceutical Design. 15(16): 1854-1867.

Gamlem H and Nordstoga K 2008. Canine vascular neoplasia – histologic classification and immunohistochemical analysis of 221 tumours and tumour-like lesions. APMIS. 116: 19-40.

Ganter MT, Cohen MJ, Brohi K, Chesebro BB, Staudenmayer KL, Rahn P, Christiaans SC, Bir ND and Pittet JF 2008. Angiopoietin-2, marker and mediator of endothelial activation with prognostic significance early after trauma? Ann Surg. 247(2): 320-326.

Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D and Betsholtz C 2003. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J Cell Biol. 161(6): 1163-1177.

Goldschmidt MH and Hendrick MJ 2008. Tumors of the Skin and Soft Tissues. In: Tumors in Domestic Animals. Iowa State Press. 45-117.

Goritz M, Muller K, Krastel D, Staudacher G, Schmidt P, Kuhn M, Nickel R and Schoon HA 2013. Canine splenic haemangiosarcoma: influence of metastases,

chemotherapy and growth pattern on post-splenectomy survival and expression of angiogenic factors. J Comp Pathol. 149(1): 30-39.

Hadem J, Bockmeyer CL, Lukasz A, Pischke S, Schneider AS, Wedemeyer H, Jonigk D, Manns MP and Kumpers P 2012. Angiopoietin-2 in acute liver failure. Crit Care Med. 40(5): 1499-1505.

Hanahan D 1997. Signaling vascular morphogenesis and maintenance. Science. 277(5322): 48-50.

Hanahan D and Weinberg RA 2011. Hallmarks of cancer: the next generation. Cell. 144(5): 646-674.

Hawighorst T, Skobe M, Streit M, Hong YK, Velasco P, Brown LF, Riccardi L, Lange-Asschenfeldt B and Detmar M 2002. Activation of the tie2 receptor by angiopoietin-1 enhances tumor vessel maturation and impairs squamous cell carcinoma growth. Am J Pathol. 160(4): 1381-1392.

Helfrich I, Edler L, Sucker A, Thomas M, Christian S, Schadendorf D and Augustin HG 2009. Angiopoietin-2 levels are associated with disease progression in metastatic malignant melanoma. Clin Cancer Res. 15(4): 1384-1392.

Hida K, Ohga N, Akiyama K, Maishi N and Hida Y 2013. Heterogeneity of tumor endothelial cells. Cancer Science. 104(11): 1391-1395.

Holopainen T, Saharinen P, D'Amico G, Lampinen A, Eklund L, Sormunen R, Anisimov A, Zarkada G, Lohela M, Helotera H, Tammela T, Benjamin LE, Yla-Herttuala S, Leow CC, Koh GY and Alitalo K 2012. Effects of angiopoietin-2-blocking antibody on endothelial cell-cell junctions and lung metastasis. J Natl Cancer Inst. 104(6): 461-475.

Jakab C, Halasz J, Kiss A, Schaff Z, Rusvai M, Galfi P, Abonyi TZ and Kulka J 2009. Claudin-5 protein is a new differential marker for histopathological differential diagnosis of canine hemangiosarcoma. Histol Histopathol. 24(7): 801-813.

Jeng MR, Fuh B, Blatt J, Gupta A, Merrow AC, Hammill A and Adams D 2014. Malignant transformation of infantile hemangioma to angiosarcoma: response to chemotherapy with bevacizumab. Pediatr Blood Cancer. 61(11): 2115-2117.

Kirby GM, Mackay A, Grant A, Woods P, McEwen B, Khanna C, Macri J, Hayes MA and Stalker M 2011. Concentration of Lipocalin Region of Collagen XXVII Alpha 1 in

the Serum of Dogs with Hemangiosarcoma. Journal of Veterinary Internal Medicine. 25(3): 497-503.

Kopczyńska E, Makarewicz R, Biedka M, Kaczmarczyk A, Kardymowicz H and Tyrakowski T 2009. Plasma concentration of angiopoietin-1, angiopoietin-2 and Tie-2 in cervical cancer. European journal of gynaecological oncology. 30(6): 646-649.

Lee HJ, Cho CH, Hwang SJ, Choi HH, Kim KT, Ahn SY, Kim JH, Oh JL, Lee GM and Koh GY 2004. Biological characterization of angiopoietin-3 and angiopoietin-4. Faseb j. 18(11): 1200-1208.

Locke JE and Barber LG 2006. Comparative aspects and clinical outcomes of canine renal hemangiosarcoma. J Vet Intern Med. 20(4): 962-967.

Maxie MG 2007. Pathology of Domestic Animals. In: Elsevier Saunders.

Mazzieri R, Pucci F, Moi D, Zonari E, Ranghetti A, Berti A, Politi LS, Gentner B, Brown JL, Naldini L and De Palma M 2011. Targeting the ANG2/TIE2 axis inhibits tumor growth and metastasis by impairing angiogenesis and disabling rebounds of proangiogenic myeloid cells. Cancer Cell. 19(4): 512-526.

Moore A, Madewell B and Lund J 1989a. Immunohistochemical evaluation of intermediate filament expression in canine and feline neoplasms. American journal of veterinary research. 50(1): 88-92.

Moore AS, Madewell BR and Lund JK 1989b. Immunohistochemical evaluation of intermediate filament expression in canine and feline neoplasms. Am J Vet Res. 50(1): 88-92.

Moreira IS, Fernandes PA and Ramos MJ 2007. Vascular endothelial growth factor (VEGF) inhibition--a critical review. Anticancer Agents Med Chem. 7(2): 223-245.

Nathenson MJ, Molavi D and Aboulafia A 2014. Angiosarcoma Arising in a Patient with a 10-Year-Old Hemangioma. Case Reports in Oncological Medicine. 2014: 5.

Neuhauser TS, Derringer GA, Thompson LDR, Fanburg-Smith JC, Miettinen M, Saaristo A and Abbondanzo SL 2000. Splenic Angiosarcoma: A Clinicopathologic and Immunophenotypic Study of 28 Cases. Mod Pathol. 13(9): 978-987.

Oksanen A 1978. Haemangiosarcoma in dogs. J Comp Pathol. 88(4): 585-595.

Paik HH and Komorowski R 1976. Hemangiosarcoma of the abdominal wall following irradiation therapy of endometrial carcinoma. Am J Clin Pathol. 66(5): 810-814.

Park YS, Kim NH and Jo I 2003. Hypoxia and vascular endothelial growth factor acutely up-regulate angiopoietin-1 and Tie2 mRNA in bovine retinal pericytes. Microvasc Res. 65(2): 125-131.

Partanen J, Puri MC, Schwartz L, Fischer KD, Bernstein A and Rossant J 1996. Cell autonomous functions of the receptor tyrosine kinase TIE in a late phase of angiogenic capillary growth and endothelial cell survival during murine development. Development. 122(10): 3013-3021.

Puri MC and Bernstein A 2003. Requirement for the TIE family of receptor tyrosine kinases in adult but not fetal hematopoiesis. Proc Natl Acad Sci U S A. 100(22): 12753-12758.

Risau W and Lemmon V 1988. Changes in the vascular extracellular matrix during embryonic vasculogenesis and angiogenesis. Dev Biol. 125(2): 441-450.

Sabattini S and Bettini G 2009. An Immunohistochemical Analysis of Canine Haemangioma and Haemangiosarcoma. Journal of Comparative Pathology. 140(2–3): 158-168.

Saharinen P, Kerkelä K, Ekman N, Marron M, Brindle N, Lee GM, Augustin H, Koh GY and Alitalo K 2005. Multiple angiopoietin recombinant proteins activate the Tie1 receptor tyrosine kinase and promote its interaction with Tie2. The Journal of Cell Biology. 169(2): 239-243.

Sallinen H, Heikura T, Laidinen S, Kosma VM, Heinonen S, Yla-Herttuala S and Anttila M 2010. Preoperative angiopoietin-2 serum levels: a marker of malignant potential in ovarian neoplasms and poor prognosis in epithelial ovarian cancer. Int J Gynecol Cancer. 20(9): 1498-1505.

Schultheiss PC 2004. A retrospective study of visceral and nonvisceral hemangiosarcoma and hemangiomas in domestic animals. J Vet Diagn Invest. 16(6): 522-526.

Sfiligoi C, de Luca A, Cascone I, Sorbello V, Fuso L, Ponzone R, Biglia N, Audero E, Arisio R, Bussolino F, Sismondi P and De Bortoli M 2003. Angiopoietin-2 expression in breast cancer correlates with lymph node invasion and short survival. Int J Cancer. 103(4): 466-474.

Shim WSN, Ho IAW and Wong PEH 2007. Angiopoietin: A TIE(d) Balance in Tumor Angiogenesis. American Association for Cancer Research. 5(7): 655-665.

Sorenmo K, Duda L, Barber L, Cronin K, Sammarco C, Usborne A, Goldschmidt M and Shofer F 2000. Canine Hemangiosarcoma Treated with Standard Chemotherapy and Minocycline. Journal of Veterinary Internal Medicine. 14(4): 395-398.

Spangler WL and Kass PH 1997. Pathologic factors affecting postsplenectomy survival in dogs. J Vet Intern Med. 11(3): 166-171.

Tabruyn SP, Colton K, Morisada T, Fuxe J, Wiegand SJ, Thurston G, Coyle AJ, Connor J and McDonald DM 2010. Angiopoietin-2-Driven Vascular Remodeling in Airway Inflammation. The American Journal of Pathology. 177(6): 3233-3243.

Tadros A, Hughes DP, Dunmore BJ and Brindle NP 2003. ABIN-2 protects endothelial cells from death and has a role in the antiapoptotic effect of angiopoietin-1. Blood. 102(13): 4407-4409.

Thurston G 2003. Role of Angiopoietins and Tie receptor tyrosine kinases in angiogenesis and lymphangiogenesis. Cell Tissue Res. 314(1): 61-68.

Thurston G, Suri C, Smith K, McClain J, Sato TN, Yancopoulos GD and McDonald DM 1999. Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. Science. 286(5449): 2511-2514.

Valenzuela DM, Griffiths JA, Rojas J, Aldrich TH, Jones PF, Zhou H, McClain J, Copeland NG, Gilbert DJ, Jenkins NA, Huang T, Papadopoulos N, Maisonpierre PC, Davis S and Yancopoulos GD 1999. Angiopoietins 3 and 4: diverging gene counterparts in mice and humans. Proc Natl Acad Sci U S A. 96(5): 1904-1909.

van der Heijden M, van Nieuw Amerongen GP, Chedamni S, van Hinsbergh VW and Johan Groeneveld AB 2009. The angiopoietin-Tie2 system as a therapeutic target in sepsis and acute lung injury. Expert Opin Ther Targets. 13(1): 39-53.

von Beust BR, Suter MM and Summers BA 1988. Factor VIII-related antigen in canine endothelial neoplasms: an immunohistochemical study. Vet Pathol. 25(4): 251-255.

Wada T, Jesmin S, Gando S, Yanagida Y, Mizugaki A, Sultana SN, Zaedi S and Yokota H 2012. Angiogenic factors and their soluble receptors predict organ dysfunction and mortality in post-cardiac arrest syndrome. Critical Care. 16(5): R171-R171.

Wang ZQ, Sun XL, Su HL, Liu XF, Xuan YJ and Yu SQ 2015. Association between serum angiopoietin-2 concentration and clinicopathological parameters in patients with colorectal cancer. Genet Mol Res. 14(4): 15547-15552.

Ward H, Fox LE, Calderwood-Mays MB, Hammer AS and Couto CG 1994. Cutaneous hemangiosarcoma in 25 dogs: a retrospective study. J Vet Intern Med. 8(5): 345-348.

Willcox TM, Speer RW, Schlinkert RT and Sarr MG 2000. Hemangioma of the spleen: Presentation, diagnosis, and management. Journal of Gastrointestinal Surgery. 4(6): 611-613.

Withrow SJ and MacEwan EG 2001. Small Animal Clinical Oncology. In: W. B. Saunders.

Withrow SJ, Page R and Vail DM 2013. Withrow and MacEwen's Small Animal Clinical Oncology. In: Elsevier Health Sciences.

Wood C, Moore A, Gliatto J, Ablin L, Berg R and Rand W 1998. Prognosis for dogs with stage I or II splenic hemangiosarcoma treated by splenectomy alone: 32 cases (1991-1993). Journal of the American Animal Hospital Association. 34(5): 417-421.

Yuan HT, Khankin EV, Karumanchi SA and Parikh SM 2009. Angiopoietin 2 is a partial agonist/antagonist of Tie2 signaling in the endothelium. Mol Cell Biol. 29(8): 2011-2022.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

APPENDIX

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Appendix 1. Ang-2 concentration

No.	Histopathological diagnosis	Ang-2 concentration (pg/ml)	
		Before surgery	After surgery [1]
1	HSA	1,500	1,600
2	HSA	2,500	5,000
3	HSA with Melanoma	6,000	10,000
4	HSA	1,250	800
5	HSA	1,100	N/A
6	HSA	1,400	N/A
7	HSA with Sertoli cell tumor	7,700	N/A
8	HSA	3,000	N/A
9	HSA	1,562	1,560
10	HSA	1,100	1,250
11	HSA	2,000	N/A
12	HSA	1,400	N/A
13	HSA with adenocarcinoma	600	1,050
14	Hemangioma	2,000	N/A
15	Hemangioma	11,800	N/A
16	Hemangioma	1,450	1,500
17	Hemangioma	7,500	N/A
18	Hemangioma	7,000	11,000
19	Hemangioma	2,700	1,562
20	Lymphoma	1,500	750
21	Lymphoma	1,562	N/A
22	Lymphoma	3,125	N/A
23	Lymphoma	1,56	N/A
24	Hematoma	1,000	1,500
25	Hematoma	8,500	N/A
26	Splenic nodular hyperplasia	2,250	2,500

Chulalongkorn University

No.	Histopathological diagnosis	Ang-2 concentration (pg/ml)	
		Before surgery	After surgery [1]
27	Splenic nodular hyperplasia	1,050	1,450
28	Splenic nodular hyperplasia	950	1,150
29	Malignant fibrous histiocytic sarcoma	1,150	3,125
30	Splenic plasma cell tumor	2,000	1,450
31	Histiocytoma	1,350	N/A
32	Splenic infarction	6,300	N/A
33	Splenic congestion	1,550	N/A
34	MCTs (Metastasis)	2,250	4,500
35	Metastatic granulosa cell tumor	1,400	1,400
36	Plamacytic splenitis	6,000	N/A
37	Histiocytic splenitis	9,000	8,000
38	Histiocytic splenitis	4,000	N/A
39	Supperative splenitis	1,100	N/A
40	Necrotic splenitis	4,500	N/A
41	Normal	900	1,350
42	Normal	700	1,610
43	Normal	5,750	1,200
44	Normal	3,000	6,000
45	Normal	1000	1,100
46	Normal	1,050	1,350
47	Normal	2,750	4,000
48	Normal	1,100	1,400
49	Normal	500	800
50	Normal	3,250	5,500

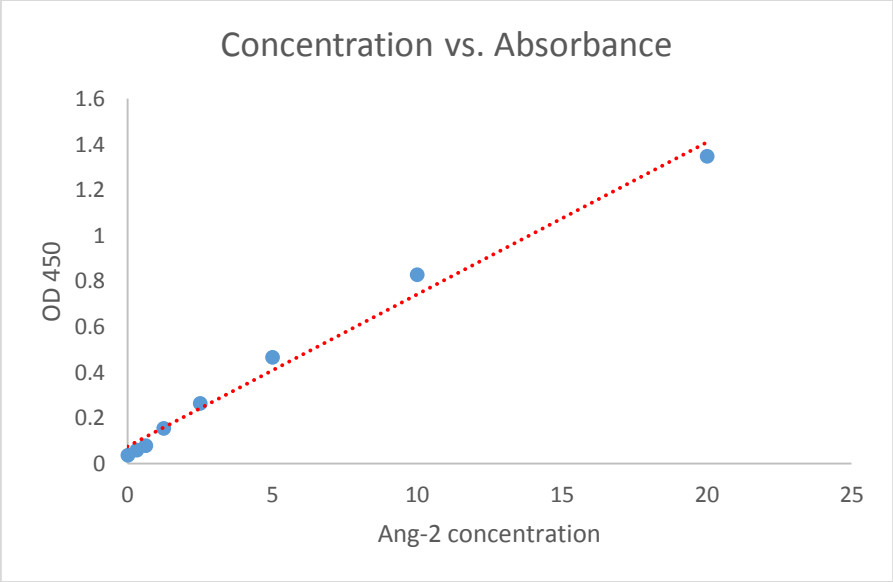
[1]N/A, unknown

Chulalongkorn University

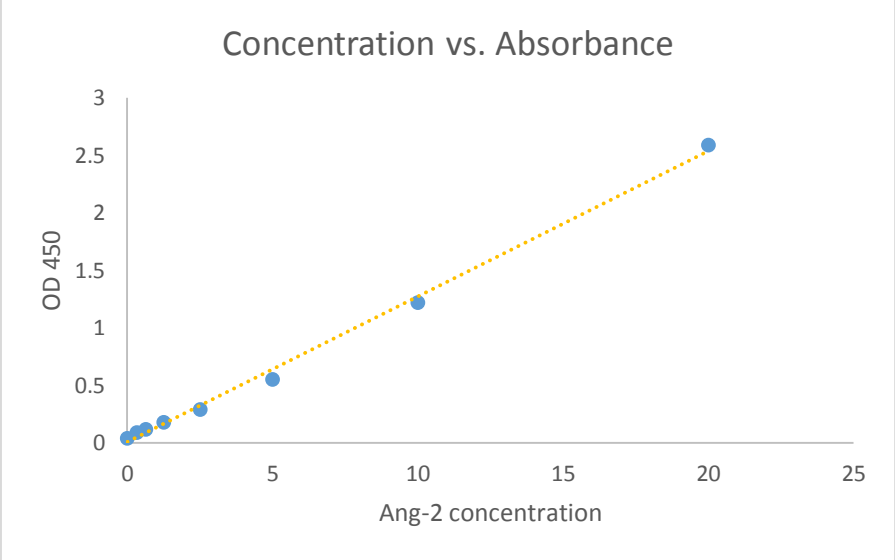
Appendix 2. Microscopic features of the dogs with splenic HSA

No.	Necrosis	Hemorrhage	Hemosiderin	Hemophagcytosis	Thrombosis	Mitotic figure	Nuclear atypia	Vascular space	Extramedullary hematopoiesis
1.	Yes	Yes	Yes	No	No	No	severe	Slit	Yes
2.	Yes	Yes	Yes	No	No	Yes	severe	No	Yes
3.	No	Yes	Yes	No	No	Yes	moderate	Honeycomb	Yes
4.	Yes	Yes	Yes	No	No	Yes	severe	No	Yes
5.	No	Yes	Yes	No	No	Yes	severe	Honeycomb	Yes
6.	No	Yes	Yes	No	Yes	No	Moderate	Honeycomb	Yes
7.	No	Yes	Yes	No	No	No	Mild	Honeycomb	No
8.	Yes	Yes	Yes	Yes	Yes	Yes	Severe	Honeycomb	No
9.	Yes	Yes	Yes	Yes	Yes	Yes	Mild	Honeycomb	Yes
10.	Yes	Yes	Yes	No	No	No	Severe	Honeycomb	Yes
11.	Yes	Yes	No	Yes	No	Yes	Severe	No	No
12.	Yes	Yes	Yes	No	Yes	No	Moderate	Honeycomb	Yes
13.	No	Yes	Yes	No	Yes	No	Mild	Honeycomb	Yes

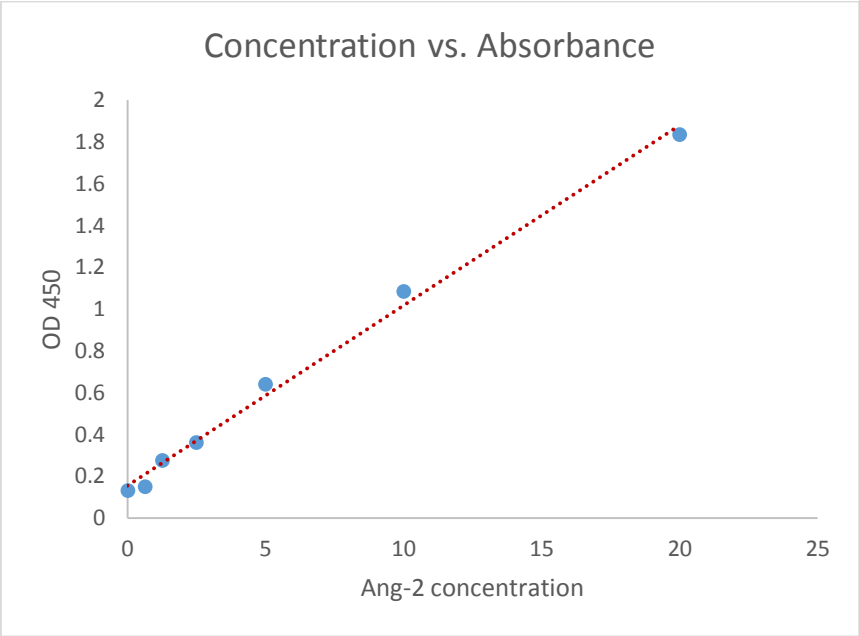
Appendix 3. Standard curve of Ang-2



Lot# 101203KB, Cat# BG-CAN10138



Lot# 032304KB, Cat# BG-CAN10138



Lot# 070301SP, Cat# BG-CAN10138

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

VITA

Miss Supissara Wongsuttawas was born on June 4th, 1987 in Khonkaen province, Thailand. She earned her bachelor degree in Doctor of Veterinary Medicine (D.V.M.); second-class honor, from the Faculty of Veterinary Science, Chulalongkorn University, Thailand, in 2010. After graduation, she had spent 3 years as a clinician in a private small animal hospital. In 2013, she enrolled the Master Degree Program in Veterinary Surgery, Faculty of Veterinary Science, Chulalongkorn University.