HOKKAIDO UNIVERSITY

Title	The study on diagnosis and clinical aspects of focal liver lesions in dogs [an abstract of entire text]
Author(s)	Leela-arporn, Rommaneeya
Description	この博士論文全文の閲覧方法については、以下のサイトをご参照ください。 https://www.lib.hokudai.ac.jp/dissertations/copy-guides/
Degree Grantor	北海道大学
Degree Name	博士(獣医学)
Dissertation Number	甲第13725号
Issue Date	2019-09-25
Doc URL	https://hdl.handle.net/2115/76395
Type	doctoral thesis
File Information	Rommaneeya_LEELA-ARPORN_summary.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

The study on diagnosis and clinical aspects of focal liver lesions in dogs

（犬の肝局所性病変の診断ならびに臨床的研究）

Rommaneeya Leela-arporn

Laboratory of Veterinary Internal Medicine

Department of Veterinary Clinical Sciences

Graduate School of Veterinary Medicine

Hokkaido University

September 2019

The study on diagnosis and clinical aspects of focal liver lesions in dogs

(犬の肝局所性病変の診断ならびに臨床的研究)

Rommaneeya Leela-arporn

GENERAL ABBREVIATIONS

ACVIM	American College of Veterinary Internal Medicine
ACTH	adrenocorticotrophic hormone
Alb	albumin
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
AUC	area under the curve
CI	confidence interval
FLL	focal liver lesion
GGT	gamma-glutamyl transferase
Glc	glucose
HCC	hepatocellular carcinoma
HCT	hematocrit
HUVTH	Hokkaido University Veterinary Teaching Hospital
NASH	non-alcoholic steatohepatitis
NPV	negative predictive value
OR	odds ratio
PLT	platelet
PPV	positive predictive value
PU/PD	polyuria and polydipsia
ROC	receiver operating characteristic
T-bil	total bilirubin
tCa	total calcium

TCho	total cholesterol
TG	triglyceride
TP	total protein
TPO	thrombopoietin
US	ultrasonography
VH	vacuolar hepatopathy
WBC	white blood cell
WSAVA	World Small Animal Veterinary Association

TABLE OF CONTENTS

GENERAL INTRODUCTION.....1

CHAPTER 1

PREDICTIVE FACTORS OF MALIGNANCY IN DOGS WITH FOCAL LIVER LESIONS USING CLINICAL DATA AND ULTRASONOGRAPHIC FEATURES.....4

1. INTRODUCTION 5

2. MATERIALS AND METHODS.....6

2.1. Study population.....6

2.2. Data collection.....6

2.3. Statistical analysis 7

3. RESULTS 9

3.1. Animals 9

3.2. Histopathologic classification 9

3.3. Predictive factors of liver malignancy.....10

4. DISCUSSION.....17

5. SUMMARY.....21

CHAPTER 2

EPIDEMIOLOGY OF MASSIVE HEPATOCELLULAR CARCINOMA IN DOGS: A 4-YEAR RETROSPECTIVE STUDY 22

1. INTRODUCTION 23

2. MATERIALS AND METHODS.....24

2.1. Study population....24
2.2. Data collection....24
2.3. Statistical analysis....26
3. RESULTS....27
3.1. Prevalence estimates....27
3.2. Risk factors for HCC....27
3.3. Clinical characteristics of HCC....28
4. DISCUSSION....34
5. SUMMARY....38

GENERAL CONCLUSION....39
JAPANESE SUMMARY....41
REFERENCES....44
ACKNOWLEDGEMENTS....51

GENERAL INTRODUCTION

Focal liver lesions (FLLs) that present as nodules or masses in dogs may be relatively common findings. Relevant clinical signs would be the reason for the visit an animal hospital. FLLs could be benign liver structural changes or malignant liver tumors. Generally, liver tumor in dogs, which can be both primary and metastatic, are usually malignant. Primary liver tumors are relatively rare, accounting for 0.6 to 1.3%[1] of all tumors in dogs. The most common primary liver tumor in dogs is hepatocellular carcinoma (HCC).[1-3] However, an appropriate management for the lesion depends on the diagnosis which could be benign or malignant pathologic conditions. Therefore, it is important to gain a tentative diagnosis of the lesion before surgical treatment. Unfortunately, liver biopsy, which is the gold standard for a definitive diagnosis of the lesion types[4], is invasive and can cause life-threatening complications as consequences.[5,6] Thus, non-invasive diagnostic methods for determining the nature and importance of FLLs are needed.

To the best of our knowledge, FLLs generally cannot be diagnosed by clinical signs, blood examination, or abdominal radiography; however, they are easily detected using current diagnostic imaging methods, including abdominal ultrasonography (US), resulting in an increase in the number of animals in which FLLs are incidentally discovered.

Recent advances in diagnostic technology which is a new US technique called contrast-enhanced US, can provide real-time perfusion imaging of many organs[7], has been mainly used in investigating FLLs in dogs[7,8] due to its diagnostic capability to differentiate benign and malignant FLLs with high accuracy.[8] However, this technique is only available in a limited number of countries due to local regulations and the need for specific equipment, including contrast agent, transducers, and special software for analysis. Therefore, attempting to use applicable characteristics of the FLLs based on current diagnostic imaging methods would

serve as valuable methods for distinguishing benign from malignant liver lesion and could help clinicians in making decision for a treatment plan, although imaging diagnosis remains challenging for predicting liver malignancy.

Conventional B-mode US is a simple diagnostic method commonly used in clinical settings to investigate the liver by evaluating its appearance to detect lesions that affect the liver parenchyma.[9,10] Unfortunately, it is widely known that the US characteristics of FLLs cannot provide a specific diagnosis.[11-15] Moreover, clinical data, including signalment, clinical signs, and laboratory findings, are generally considered nonspecific findings. However, a combination of clinical data and US features of FLLs may allow prediction of whether lesions are benign or malignant.

Besides the challenge of diagnostic technology for distinguishing pathologic varieties of FLLs, little is known regarding epidemiological features of HCC in dogs which is the most common primary liver tumor. In humans, the development of HCC is associated with major risk factors, including cirrhosis, chronic infection with hepatitis B and C viruses, alcoholic fatty liver disease, and non-alcoholic fatty liver disease. However, similar risk factors have not been identified in dogs because a viral aetiology has not been detected in dogs, and an association between cirrhosis and HCC in dogs is rare, representing only 7% of dogs with HCC.[1,17]

A few studies have explored the risk factors for HCC in dogs.[1,2,18,19] However, the clinical features and risk factors for HCC in dogs have not yet been confirmed. In addition, previous studies have reported that vacuolar hepatopathy (VH) in Scottish Terriers may be associated with HCC development, suggesting that VH might be a risk factor for HCC.[20-22] Therefore, it is possible that VH-related disorders can increase the risk of HCC development.[22] However, a search for concurrent disorders in dogs with HCC has not been performed.

With the above background, the aims of this study were to investigate the clinical utility of current diagnostic methods in distinguishing pathologic varieties of FLLs whether lesions

are more likely to be attribute to benign or malignant, and in order to gain new insight into the potential factors associated with HCC in dogs. This study was specifically focused on 2 parts including diagnosis of FLLs and clinical aspects of HCC in dogs. In chapter 1, the diagnostic performance of clinical data and US appearances of dogs with FLLs were determined for predicting liver malignancy. In chapter 2, the prevalence and potential risk factors associated with HCC in dogs were investigated.

CHAPTER 1

PREDICTIVE FACTORS OF MALIGNANCY IN DOGS WITH FOCAL LIVER LESIONS USING CLINICAL DATA AND ULTRASONOGRAPHIC FEATURES

1. INTRODUCTION

An FLL that presents in dogs can be either a benign or a malignant liver disease. A definitive diagnosis of FLLs requires invasive procedures for histopathologic examination[4], which is expensive and invasive, and it can result in life-threatening complications.[5,6] Thus, noninvasive diagnostic methods for determining the nature and importance of FLLs are needed.

Recently, contrast-enhanced US, an advanced diagnostic technique, has become increasing popularity for examining FLLs due to its ability in distinguishing benign and malignant FLLs with high accuracy.[7,8] However, this technique may not be easily accessible due to the cost, the needs for specific equipment, and their limited availability in veterinary facilities. Therefore, a simpler and noninvasive diagnostic method for distinguishing benign from malignant FLLs is needed.

Conventional B-mode US is a simple diagnostic method commonly used in clinical settings to investigate the liver by evaluating its appearance, including the echogenicity, echotexture, size, shape and margins, and to detect lesions that affect the liver parenchyma.[9,10] However, it remains diagnostically challenging to determine the nature of FLLs based solely on this method due to overlap of the US features of malignant and benign liver lesions.[10,15] Recent studies have conversely suggested that several US features of FLLs may be related to malignant conditions and liver cytology results.[16,23,24] In addition, clinical data, including signalment, clinical signs, and laboratory findings, are generally considered nonspecific findings. The use of this information alone is not sufficiently accurate to determine the causes of FLLs.[3,11-15] Therefore, a combination of clinical data and US features of FLLs may have the potential to predict pathologic types of liver lesions.

The goal of chapter 1 was to determine the clinical relevance of clinical and US data for the prediction of liver malignancy in dogs.

2. MATERIALS AND METHODS

2.1. Study population

A retrospective study was conducted using information from dogs with FLLs with histologically confirmed diagnoses between January 2013 and July 2018 at Hokkaido University Veterinary Teaching Hospital (HUVTH). The inclusion criteria of this study were dogs with FLLs that underwent abdominal US and histopathologic examinations following surgery or liver biopsy. All of the histopathologic examinations were performed by a board-certified pathologist.

Dogs were excluded from this study if they did not undergo abdominal US examination, if they had no representative US images of the liver, or if the quality of US images was poor due to the possibility of misinterpretation.

2.2. Data collection

Medical records were reviewed for candidate predictive factors, including signalment, clinical signs, clinicopathologic findings, and abdominal US findings. Signalment consisted of age, body weight and sex. Clinical signs consisted of anorexia, weight loss, lethargy, polyuria and polydipsia (PU/PD), vomiting, diarrhea, jaundice, and neurological signs.

Clinicopathologic findings, including hematologic and serum biochemical analyses, were extracted from the medical records of all of the included dogs over a 2-week period of abdominal US examinations. Hematological abnormalities were defined as follows: leukocytosis, white blood cell (WBC) count >17 × 10^3 cells/μL (reference range, 6-17 ×10^3 cells/μL); anemia, hematocrit (HCT) <37% (reference range, 37-55%); and thrombocytosis, platelet (PLT) count >500 × 10^3 cells/μL (reference range, 200-500 ×10^3 cells/μL). The upper limits of the reference ranges for liver enzymes, including serum alkaline phosphatase (ALP),

alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transferase (GGT) activities, were 254 IU/L (reference range, 47-254 IU/L), 78 IU/L (reference range, 17-78 IU/L), 44 IU/L (reference range, 17-44 IU/L), and 14 IU/L (reference range, 5-14 IU/L), respectively. The reference range for the total bilirubin (T-bil) concentration was 0.1-0.5 mg/dL.

All of the US images were collected using one of three US scanners (Aplio XG and Aplio 500, Toshiba Medical Systems, Tochigi, Japan; HI VISION Preirus, Hitachi Medical Corp, Chiba, Japan) that are available in the HUVTH. The US findings of FLLs included maximum size, number, margin, echotexture, and echogenicity relative to the liver parenchyma. These US findings were compared with histopathologic results as predictors of malignant and benign liver diseases. The size of FLLs was defined as the maximum diameter based on the maximum measurable diameter in each lesion. The number of FLLs was recorded as single or multiple. The margin of FLLs was categorized as smooth or irregular. Additionally, the echotexture of FLLs was classified by its uniformity as homogeneous or heterogeneous throughout the lesion's parenchyma. The echogenicity of FLLs was categorized as anechoic, hypoechoic, isoechoic, hyperechoic, or mixed echogenicity from the lesion's brightness relative to the surrounding liver parenchyma. The presence or absence of peritoneal fluid, hepatic lymphadenopathy, and calcification was also evaluated. All of the US images were assessed using medical imaging viewer software (OsiriX, Pixmeo SARL, Bernex, Switzerland) as a reference for FLLs by two investigators (RL and MT).

2.3. Statistical analysis

Comparisons of all of the predictive factors of benign and malignant liver lesions were conducted with univariate analyses using Fisher's exact test or the chi-square test for categorical variables, including sex, the presence of clinical signs, the presence of abnormal

clinicopathologic findings, US appearance of FLLs, and the presence of ascites, hepatic lymphadenopathy, and calcification. The data are presented as numbers and percentages. Continuous variables, including age, body weight and maximum lesion size, were assessed using the Mann-Whitney U-test and are expressed as the medians and ranges. Spearman's correlation analysis was performed to evaluate the possible relationship between the body weights of dogs and the lesion size. The optimal cut-off values of lesion size to predict malignancy were chosen from a receiver operating characteristic (ROC) curve analysis with the criterion variables "maximum lesion size" and "malignant" as condition variables.

A multivariable logistic regression model was used to select predictive factors from univariate analyses via a forward stepwise selection procedure. The selection used a threshold *P* value ($P < 0.15$ for inclusion, $P > 0.2$ for exclusion) to identify independent predictors with the strongest associations with liver malignancy. Then, the odds ratio (OR) and 95% confidence interval (CI) of each predictive variable that was included in the multivariate model were calculated. The diagnostic accuracy of the predictive model of independent predictors was assessed by a ROC curve. For all of the statistical analyses, a *P* value < 0.05 was considered significant. All of the data were analyzed using commercial statistical software (JMP Pro, version 14.0.0, SAS Institute Inc.).

3. RESULTS

3.1. Animals

A total of 91 dogs with histopathologic diagnoses of FLLs were identified during the study period. A total of 83 dogs met the inclusion criteria. The remaining 8 dogs were excluded due to poor US image quality or inadequately representative US images (Figure 1).

Of these 83 dogs, the dog breeds included 13 Miniature Dachshunds, 8 Chihuahuas, 6 Beagles, 6 Welsh Corgis, 6 Shiba Inus, 6 Yorkshire Terriers, 5 Shih Tzus, 4 Toy Poodles, 3 Golden Retrievers, 3 Labrador Retrievers, 3 Mongrels, 2 American Cocker Spaniels, 2 Border Collies, 2 Malteses, 2 Miniature Schnauzers, 2 Papillons, and one each of the following: Boston Terrier, Cairn Terrier, French Bulldog, Jack Russel, Lhasa Apso, Pekingese, Pug, Scottish Terrier, Shetland Sheepdog, and Standard Dachshund.

3.2. Histopathologic classification

Histopathologic results revealed that 55 dogs had malignant lesions, and 28 dogs had benign lesions. Of the malignant lesions, there were 37 HCCs, five hemangiosarcomas, four undifferentiated sarcomas, three cholangiocellular carcinomas, three hepatocholangiocellular carcinomas, and three metastatic lesions. Benign lesions included 12 nodular hyperplasias, six glycogen accumulations, three lesions of cholangiohepatitis, two normal livers, and one each of hepatitis, amyloidosis, hepatic cyst, biliary cyst, and hematoma.

3.3. Predictive factors of liver malignancy

The evaluation of the predictive factors possibly associated with liver malignancy was performed by univariate analyses of clinical data and US features. The results of all of the predictive factors are summarized in Table 1.

Regarding predictive factors of clinical data, the median ages of dogs with benign and malignant liver lesions were 11 years (range, 6-17 years) and 12 years (range, 7-16 years), respectively, which were not significantly different ($P = 0.8956$). There was also no significant difference between the body weights of dogs between benign and malignant liver lesions ($P = 0.4671$), and the median body weights of dogs with benign and malignant lesions were 7.4 kg (range, 2.3-22.9) and 7.5 kg (range, 1.7-37), respectively. Thirteen male and 15 female dogs had benign lesions, and 32 male and 23 female dogs had malignant lesions. The sex distributions were not significantly different between dogs with benign and malignant liver lesions ($P = 0.3565$). Of these 83 dogs, only 41 dogs, including 13 dogs with benign liver lesions and 28 dogs with malignant liver lesion, presented with clinical signs; however, no significant differences were found regarding the presence of clinical signs, as shown in Table 1. For the hematologic findings, data were extracted from the medical records of 83 dogs to obtain HCT data and from 82 dogs to obtain WBC and PLT counts. For serum biochemical findings, data were extracted from the medical records of 83 dogs to obtain ALP and ALT levels, 61 dogs to obtain AST levels, 47 dogs to obtain GGT levels, and 75 dogs to obtain T-bil levels. Among the clinical data, the results of univariate analyses indicated that the PLT count was the only factor predictive of liver malignancy in which dogs with malignant liver lesions significantly represented with thrombocytosis ($P = 0.0169$).

Three US variables were significantly different between benign and malignant liver lesions. The maximum lesion size of malignant liver lesions (median 5.1 cm, range: 0.9-15.3 cm) was significantly larger than that of benign liver lesions (median 1.8 cm, range: 0.4-7.0

cm) ($P < 0.0001$), and the body weight showed a positive correlation with lesion size (r = 0.1437, $P = 0.1949$). The best cut-off value of lesion size to differentiate malignant from benign liver lesions was 4.1 cm. Using the cut-off value of 4.1 cm, the diagnostic performance was as follows: accuracy: 78.3%; sensitivity: 70.9%; specificity: 92.9%; positive predictive value (PPV): 95.1%; and negative predictive value (NPV): 61.9%. In addition, compared with benign liver lesions, malignant liver lesions showed significantly heterogeneous echotexture ($P < 0.0001$) and mixed echogenicity ($P < 0.0001$) on US (Figure 2).

In the multivariate analysis, the significant predictive factors in the univariate analyses were selected using a multivariable logistic regression model. The multivariate analysis showed that the PLT count (thrombocytosis; OR: 7.17, 95% CI: 1.52-33.77, $P = 0.0127$), maximum lesion size (4.1 cm or greater; OR: 23.83, 95% CI: 3.74-151.95, $P = 0.0008$), and echotexture of FLLs (heterogeneous; OR: 8.44, 95% CI: 1.37-51.91, $P = 0.0214$) were found to be independent predictive factors of liver malignancy, as shown in Table 2. The predictive performance of this model exhibited 85.4% accuracy, 89.1% sensitivity, 77.8% specificity, 89.1% PPV, and 77.8% NPV, with an area under the curve (AUC) of 0.9185.

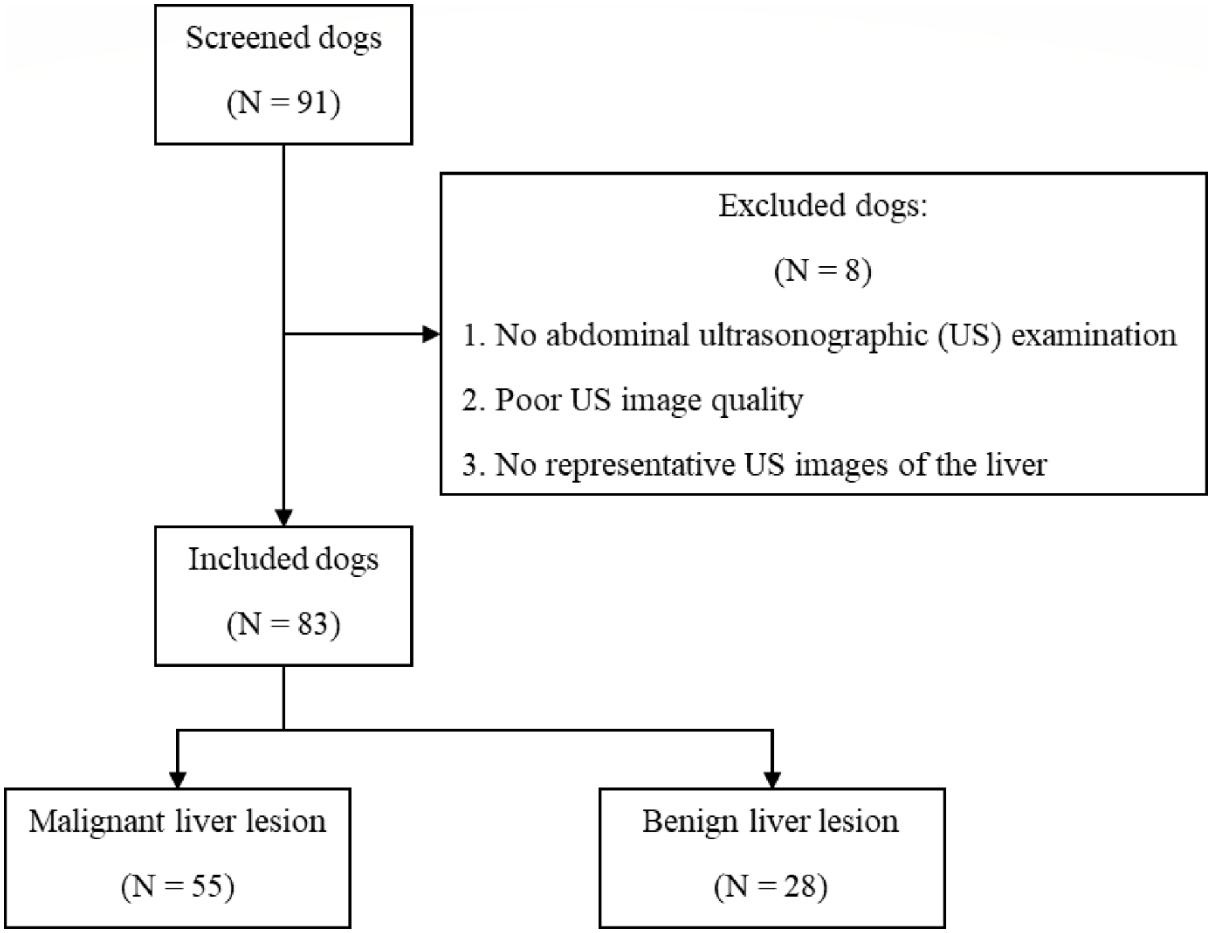

Figure 1. Diagram of patient selection.

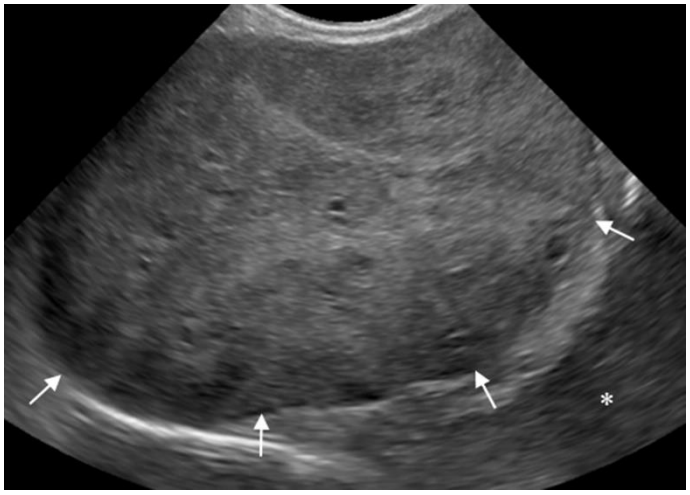

Figure 2. Conventional B-mode US image of HCC. The lesion has a heterogeneous echotexture and mixed echogenicity ranging from hypoechoic to hyperechoic (arrows), compared with the surrounding normal liver parenchyma (*).

Table 1. Comparison of the characteristics of clinical data and ultrasonographic findings between benign and malignant liver lesions in dogs.

Variable	**Total (n = 83)**		***P* value**
	Benign (n = 28)	Malignant (n = 55)	
Signalment			
Age in years – median (range)	11 (6-17)	12 (7-16)	0.8956
Body weight in kg – median (range)	7.4 (2.3-22.9)	7.5 (1.7-37)	0.4671
Sex, n (%)			0.3565
Male	13 (46.4)	32 (58.2)	
Female	15 (53.6)	23 (41.8)	
Clinical signs			
Anorexia, n (%)	6 (21.4)	12 (21.8)	1.0000
Weight loss, n (%)	2 (7.1)	6 (10.9)	0.7111
Lethargy, n (%)	5 (17.9)	9 (16.4)	1.0000
PU/PD, n (%)	5 (17.9)	8 (14.6)	0.7539
Vomiting, n (%)	3 (10.7)	3 (5.5)	0.4004
Diarrhea, n (%)	2 (7.1)	4 (7.3)	1.0000
Jaundice, n (%)	2 (7.1)	0 (0)	0.1111
Neurological signs, n (%)	1 (3.6)	0 (0)	0.3373
Clinicopathologic findings			
Leukocytosis, n (%)	6/27 (22.2)	10/55 (18.2)	0.8516
Anemia, n (%)	5/28 (17.9)	13/55 (23.6)	0.7788
Thrombocytosis, n (%)	6/27 (22.2)	30/55 (54.6)	0.0169*
High ALT level, n (%)	23/28 (82.1)	44/55 (80.0)	1.0000
High AST level, n (%)	12/21 (57.1)	17/40 (42.5)	0.2963
High ALP level, n (%)	25/28 (89.3)	47/55 (85.5)	0.7426
High GGT level, n (%)	9/18 (50.0)	11/29 (37.9)	0.5462
Hyperbilirubinemia, n (%)	4/25 (16.0)	2/50 (4.0)	0.0910

Variable	Total (n = 83)		*P* value
	Benign (n = 28)	Malignant (n = 55)	
Ultrasound			
Maximum lesion size in cm – median (range)	1.8 (0.4-7.0)	5.1 (0.9-15.3)	<0.0001*
Lesion number, n (%)			0.2288
Single	16 (57.1)	39 (70.9)	
Multiple	12 (42.9)	16 (29.1)	
Lesion margin, n (%)			0.1134
Smooth	24 (85.7)	37 (67.3)	
Irregular	4 (14.3)	18 (32.7)	
Lesion echotexture, n (%)			<0.0001*
Homogeneous	16 (57.1)	2 (3.6)	
Heterogeneous	12 (42.9)	53 (96.4)	
Lesion echogenicity, n (%)			<0.0001*
Anechoic	1 (3.6)	0 (0)	
Hypoechoic	7 (25.0)	1 (1.8)	
Hyperechoic	8 (28.6)	1 (8.2)	
Mixed echogenicity	12 (42.9)	53 (96.4)	
Ascites, n (%)	0 (0)	4 (7.3)	0.2948
Hepatic lymphadenopathy, n (%)	2 (7.1)	4 (7.3)	1.0000
Calcification, n (%)	0 (0)	0 (0)	NA

ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; NA, not assessed; PU/PD, polyuria and polydipsia.

*P values < 0.05 were statistically significant.

Table 2. Multivariable logistic regression with stepwise model selection to identify independent variables for predicting liver malignancy.

Variable	**Odds ratio**	**95% CI**	***P* value**
PLT count			
Thrombocytosis	7.17	1.52-33.77	0.0127*
Maximum lesion size			
4.1 cm in diameter or greater	23.83	3.74-151.95	0.0008*
Lesion echotexture			
Heterogeneous	8.44	1.37-51.91	0.0214*

PLT, platelet.

**P* values < 0.05 were statistically significant.

4. DISCUSSION

The goal of this retrospective study was to determine the predictive performance of clinical data and US features in determining the malignancy of FLLs. Multivariate analysis results indicated that the PLT count, maximum lesion size, and echotexture of FLLs were independent predictors for differentiating between benign and malignant liver diseases.

The results of this study revealed a heterogeneous echotexture that was significantly associated with malignant liver lesions, and this heterogeneous appearance could result from intratumoral hemorrhage and necrosis.[26] This result is consistent with the results of previous studies that described the presence of target lesions and cavitations inside a mass as signs of liver malignancy[11,16], since these 2 features also presented as heterogeneous echotexture. Thus, this result suggested that a heterogeneous echotexture of an FLL is a useful US finding for predicting malignant conditions.

However, the classification of the presence of cavitations within a mass or target lesions from a heterogeneous echotexture of FLLs did not be separately classified on US findings in the present study since this study aimed to conduct a simple US evaluation to predict benign and malignant liver lesions for clinicians to use in clinical practice; thus, the results of this study were different from those of previous studies[16,23-25] that did not show an association between the echotexture of FLLs and liver malignancies. Among the reasons for this discrepancy are the different US criteria for evaluating the appearances of FLLs[16,23-25] and different denominator populations. Furthermore, some predictive factors measured in this study were not included in previous studies.[16,23-25] Due to these differences, US classification guidelines for differentiating between benign and malignant liver lesions are needed.

The results of this study also showed that a lesion size of 4.1 cm or greater was significantly associated with malignant liver lesions, consistent with the results of previous

studies.[23,24] However, the cut-off values of maximum lesion size were greater than those of previous studies, perhaps due to the number of included dogs with HCC in this study. HCC mostly presented with large sizes of FLLs, which could have contributed to the prediction of liver malignancy based on lesion size.

The presence of ascites was not independently associated with liver malignancy, conflicting with the results of previous studies.[23,24] This discrepancy may be due to the limited number of dogs with FLLs in this study. Additionally, ascites are present not only in neoplastic diseases but also in non-neoplastic liver diseases[25], such as chronic hepatitis. In the present study, none of the benign diseases presented with ascites. Thus, the presence of ascites could have been an independent factor for predicting liver malignancy, as indicated in previous studies[23,24], had the number of dogs with FLLs been greater.

Although the clinical characteristics of dogs with FLLs are usually nonspecific[27,28], thrombocytosis was overrepresented in the dogs with malignant liver lesions examined here, possibly due to the presence of a large number of dogs with HCC in this study. This result is similar to the results of previous reports of dogs with HCC.[18] In addition, recent studies have also revealed that reactive or secondary thrombocytosis is commonly associated with neoplasias, especially carcinoma.[29-31] However, the causes of carcinoma-related thrombocytosis in dogs remain unclear; these conditions may result from paraneoplastic syndrome, as observed in human malignancies, including HCC.[32-34] In humans, tumors have been linked to the production of granulocyte-macrophage colony-stimulating factor, interleukin-6, and thrombopoietin (TPO)[33,35-37], and the liver is a source of TPO. Nevertheless, the role of TPO in liver disease in dogs has not yet been investigated, so there could be mechanisms related to thrombocytosis. Additionally, thrombocytosis can contribute to a thromboembolic event and affect prognosis, as well as survival time, as presented in humans; however, the risk of thromboembolic events, survival time or the outcomes of dogs did not be

investigated in this study. Therefore, further investigation is needed to determine the pathophysiologic mechanism of thrombocytosis and its roles as a paraneoplastic phenomenon and prognostic factor.

This study had several limitations. First, the clinical and laboratory findings could not be collected from all of the dogs included in this study. Missing data could have affected the results of the data analyses. In addition, it is possible that the presenting clinical data may not have been related to the malignant liver lesions in the enrolled dogs with multiple disease processes. Second, US assessment is subjective and depends on an observer. The observer variation could result in diagnostic variability. In this study, to minimize the variation associated with observer assessment, all of the examiners used a fixed criterion for assessment.

Third, this study used three different ultrasound devices to image FLLs. Despite this limitation, results of the present study indicated that the echotexture of FLLs could independently predict liver malignancy. Therefore, the usefulness of the US echotexture in predicting liver malignancy might not depend on the type of ultrasound device used.

Next, the body size of dogs for the lesion size variable did not be normalized due to the small effect of body weight on the lesion size variables in the present study. Thus, it is possible that there might be an effect of the body size of dogs on the liver lesion diameter. Further study is needed to confirm the effect of body weight on the lesion size of FLLs. In addition, because this study was performed at a referral hospital, there is the possibility that malignant liver lesion might be detected in lesion sizes smaller than 4.1 cm in general hospital situations.

Due to the retrospective nature of this study, another limitation is that interpretation of US appearances was performed using stored images, which might have limited the accuracy for detecting some US appearances. To minimize this limitation, video clips of the FLLs were also used to interpret US appearances.

Finally, histopathologic results were used as a reference standard and as inclusion criteria, likely leading to a number of biases since some dogs with FLLs did not undergo surgery or liver biopsy due to either the clinician's decision or the owner's personal reasons. Therefore, the sample size obtained for histopathologic examination could have affected the accuracy of the predictive model. In addition, due to the retrospective study design, this study cannot confirm that a lesion detected by US was the same lesion from which a sample was collected for histologic examination, which could have affected the accuracy of diagnosis as a limitation of clinical practice. However, since a dog may have multiple disease processes; thus, the histologic results may not have reflected the disease causing an FLL.

In conclusion, a combination of clinical and US data provides independent predictors of liver malignancy, including thrombocytosis, lesion size of 4.1 cm or greater, and heterogeneous echotexture of FLLs, that can differentiate malignant from benign liver lesions in dogs. Prediction of liver malignancy may help clinicians in clinical decision making for further examination and appropriate treatment.

5. SUMMARY

In this chapter, the clinical relevance of clinical and US data for the prediction of liver malignancy in dogs was determined. The results of univariate analyses showed that several US features and PLT count were significantly associated with liver malignancy. Multivariate analysis indicated that the PLT count, maximum lesion size, and the echotexture of FLLs were independent predictors for differentiating between benign and malignant liver diseases. Thus, a combination of clinical and US data provides independent predictors of liver malignancy, including thrombocytosis, lesion size of 4.1 cm or greater, and heterogeneous echotexture of FLLs.

CHAPTER 2

EPIDEMIOLOGY OF MASSIVE HEPATOCELLULAR CARCINOMA IN DOGS: A 4-YEAR RETROSPECTIVE STUDY

1. INTRODUCTION

Although, a few studies have explored the risk factors for HCC in dogs and have revealed that certain breeds of dogs, particularly Miniature Schnauzers and Shih Tzus, and male dogs are overrepresented for HCC.[1,2,18,19], the clinical features and risk factors of HCC in dogs have not yet been confirmed.

Previous studies have reported that VH in Scottish Terriers may be associated with HCC development, suggesting that VH might be a risk factor for HCC.[20-22] In humans, recent studies have reported that hypothyroidism and diabetes mellitus are related to HCC[38-40] due to the association with non-alcoholic steatohepatitis (NASH)[41,42], which is considered to be a predisposing condition for HCC development.[43,44]

In dogs, one previous study showed a disruption in mitochondrial ultrastructure and metabolism and modification of keratin filaments in VH livers.[22] Similar ultrastructural and metabolic changes in the liver have also been observed in humans with NASH.[45] Therefore, it is possible that VH-related disorders can increase the risk of HCC development, as 9/55 dogs with VH developed HCC.[22] However, a search for concurrent disorders in dogs with HCC has not been performed.

Due to limited information regarding the epidemiological features of HCC in dogs, the goal of chapter 2 were to estimate the prevalence of HCC and to identify potential risk factors associated with HCC, including clinicopathologic factors and concurrent disorders.

2. MATERIALS AND METHODS

2.1. Study population

A retrospective study was carried out in the HUVTH from May 2013 to May 2017. Informed consent was obtained from all owners of dogs involved in this study. Diagnosis of HCC in dogs were identified by abdominal US and histopathologic examination following surgery. All histopathologic examinations were performed by a board-certified pathologist. The pathologic diagnosis of HCC was defined according to the guidelines of the World Small Animal Veterinary Association (WSAVA) Liver Standardization Group.[46] To estimate prevalence and to examine age, sex and breed predispositions, and to investigate risk factors for HCC including concurrent disorders, all dogs presented to HUVTH during the study period were used as the reference population.

To characterize the clinical features of HCC, one-to-one propensity score matching combined with covariate adjustment was used to select a pair of dogs with and without HCC in the same conditions, resulting in no differences in age, sex, breed, and comorbidities for the case-control analysis.

2.2. Data collection

For both HCC and control dogs, data extracted from the medical records included signalment (age, sex, breed, and body weight); history of long-term steroid use in anti-inflammatory or immunosuppressive doses (0.5-2.0 mg/kg/day; ≥2 weeks)[47]; clinicopathologic findings, including hematologic and serum biochemical analyses, endocrine test results, imaging results and concurrent diseases.

Hematological abnormalities were defined as follows: leukocytosis, WBC count >17 $\times 10^3$ cells/μL (reference range, 6-17 $\times 10^3$ cells/μL); anemia, HCT <37% (reference range, 37-

55%); and thrombocytosis, PLT count > 500 $\times 10^3$ cells/μL (reference range, 200-500 $\times 10^3$ cells/μL). Serum biochemical abnormalities were defined as follows: hypoproteinemia, total protein (TP) content <5.0 g/dL (reference range, 5.0-7.2 g/dL); hypoalbuminemia, albumin (Alb) content <2.6 g/dL (reference range, 2.6-4.0 g/dL); and hypoglycemia, glucose (Glu) content <75 mg/dL (reference range, 75-128 mg/dL). The upper limits of the reference ranges for liver enzymes, including serum ALT, ALP, AST and GGT, were 78 IU/L (reference range, 17-78 IU/L), 254 IU/L (reference range, 47-254 IU/L), 44 IU/L (reference range, 17-44 IU/L) and 14 IU/L (reference range, 5-14 IU/L), respectively. In addition, hyperbilirubinemia was defined as a T-bil concentration >0.5 mg/dL (reference range, 0.1-0.5 mg/dL). Other serum biochemical abnormalities were defined as hypercalcemia, hypertriglyceridemia and hypercholesterolemia if the total calcium (tCa), triglyceride (TG) and total cholesterol (TCho) concentrations were >12.1 mg/dL (reference range, 9.3-12.1 mg/dL), >133 mg/dL (reference range, 30-133 mg/dL) and >312 mg/dL (reference range, 111-312 mg/dL), respectively.

For endocrine testing, endocrine disorders including hyperadrenocorticism, hypothyroidism and diabetes mellitus that were diagnosed at a private animal hospital or the HUVTH were considered in this study. Hyperadrenocorticism was determined if the dogs had a historical diagnosis within 6 months of HCC presentation[48], on the basis of a positive result with either a low-dose dexamethasone suppression test or an adrenocorticotrophic hormone (ACTH) stimulation test, in combination with one or more common clinical signs other than abdominal distension and hepatomegaly, as described in the consensus statement of American College of Veterinary Internal Medicine (ACVIM).[49] Diagnosis of hypothyroidism was based on a historical diagnosis of a thyroid panel and low total or free thyroxine levels with elevated thyroid-stimulating hormone levels within 6 months of HCC presentation.[48] Diabetes mellitus was considered present if the dogs had a historical diagnosis prior to or within 3 months after HCC presentation[48], based on persistent fasting hyperglycemia with clinical signs.

2.3. Statistical analysis

Period prevalence was evaluated for dogs diagnosed with HCC. Continuous variables, including age and body weight, were assessed using the Mann-Whitney U-test and were expressed as the median and range. Categorical variables, including breed, sex, clinicopathologic findings and concurrent disorders, were analyzed using Fisher's exact test or the chi-square test or. Factors possibly associated with HCC, including age, breed, sex and concurrent disorders, were assessed using univariate and multivariate logistic regression analysis. ORs and 95% CIs for univariate and multivariate associations between HCC and possible risk factors were also estimated. Statistical power analysis was conducted to determine the effect of a significant breed predisposition to HCC. Propensity score matching (1:1 match) was performed to minimize the effect of potential confounders on selection bias for case-control analysis, using multiple logistic regressions to estimate the probability of having specific clinical features for HCC. The covariates used in the propensity score were age, breed, and comorbidities. A Bonferroni correction was applied to account for the multiplicity of breeds. Statistical analyses were performed using commercial statistical software packages (JMP Pro, version 14.0.0, SAS Institute Inc., and R 3.4.1, The R Project for Statistical Computing). $P < 0.05$ was considered statistically significant ($P < 0.0036$ after Bonferroni correction).

3. RESULTS

3.1. Prevalence estimates

The study population consisted of 4,607 dogs that were presented during the study period. Forty-one dogs were diagnosed with massive-type HCC, giving a prevalence of 0.96%.

3.2. Risk factors for HCC

The ages of the dogs diagnosed with HCC (median, 11 years; range, 8-15 years) were significantly higher ($P < 0.001$) than those of the reference population (median, 9 years; range, 0-20 years). The median body weight of the dogs with HCC was 7 kg (range, 1.7-32.5 kg). The HCC group included 18 females and 26 males. Compared with each sex category in the reference population (n = 2,107 females, n = 2,456 males), there was no significant difference with the HCC group ($P = 0.3186$).

Details regarding the dog breeds are shown in Table 7. The HCC group included 7 Welsh Corgis (15.9%), 5 Beagles (11.4%), 5 Shih Tzus (11.4%), 5 Chihuahuas (11.4%), 4 Miniature Dachshunds (9.1%), 4 Yorkshire Terriers (9.1%), 4 Toy Poodles (9.1%), 4 Mongrels (9.1%) and one each of the following: Pug (2.3%), Shiba Inu (2.3%), Boston terrier (2.3%), Golden retriever (2.3%), Miniature Schnauzer (2.3%) and Pomeranian (2.3%). The total number of dogs without HCC during the study period was 4,563. Of these, 3,293 dogs belonged to one of the dog breeds in which HCC was described. The number and proportion of each breed among the dogs without HCC were as follows: 217 Welsh Corgis (4.8%), 107 Beagles (2.8%), 206 Shih Tzus (4.5%), 368 Chihuahuas (8.1%), 848 Miniature Dachshunds (18.6%), 159 Yorkshire Terriers (3.5%), 334 Toy Poodles (7.3%), 338 Mongrels (7.4%), 75 Pugs (1.6%), 178 Shiba Inus (3.9%), 36 Boston Terriers (0.8%), 93 Golden Retrievers (2%), 166 Miniature Schnauzers (3.6%) and 124 Pomeranians (2.7%). A significant breed predisposition

to HCC was observed in Welsh Corgis (OR: 3.79; 95% CI: 1.67-8.60; *P* = 0.0014) and Beagles (OR: 5.34; 95% CI: 2.06-13.81; *P* = 0.0006).

Of the 44 HCC dogs, 27 (61.4%) had at least one concurrent disease (Table 8). The most frequent concurrent disease with HCC was hyperadrenocorticism (total n = 10; n = 3 Beagles, n = 2 Chihuahuas and one each of the following: Welsh Corgi, Mongrel, Pomeranian, Boston terrier and Toy Poodle). The association of HCC with concurrent disorders, especially endocrinopathies, is shown in Table 9. Chi-square testing revealed that the OR of hyperadrenocorticism in dogs diagnosed with HCC were 6.92 times those of the controls (95% CI: 3.37-14.22; *P* < 0.0001). However, there was no significant association between HCC and hypothyroidism or diabetes mellitus (*P* > 0.05). In addition, only two HCC dogs had a history of long-term steroid use (4.5%).

Multivariate logistic regression analysis confirmed that age was significantly associated with HCC, with increased risk in older dogs (OR, 1.20; 95% CI, 1.07-1.33; *P* = 0.0005). Welsh Corgis (OR, 3.68; 95% CI, 1.56-8.67; *P* = 0.0029) and Beagles (OR, 4.33; 95% CI, 1.58-11.90; *P* = 0.0044) were the only breeds with a statistically significant predisposition to HCC (statistical power = 75.3% and 76.9%, respectively). Although Shih Tzus were a predisposed breed in univariate analysis, the statistical power was only 46%. Hyperadrenocorticism was significantly associated with HCC as a concurrent disorder (OR, 4.13; 95% CI, 1.95-8.76; *P* = 0.0002). However, sex and hypothyroidism or diabetes mellitus were not associated with HCC. Variables associated with HCC in univariate and multivariate analysis are summarized in Table 9.

3.3. Clinical characteristics of HCC

According to the one-to-one propensity score matching, 44 dogs without HCC from the reference population were matched with 44 HCC dogs. The clinicopathologic findings for the

HCC dogs were compared with those for dogs without HCC as the control group. The results of the clinicopathologic findings are summarized in Table 10. Hematology was performed in 44 dogs, and data were available for 43 dogs for WBC count, HCT and PLT count. Serum biochemical analysis was performed in 44 dogs, and ALT, ALP and TP were evaluated in all dogs. Alb and T-bil concentrations were evaluated in 42 dogs; Glu was evaluated in 41 dogs; and tCa concentrations were evaluated in 32 dogs. Serum AST and GGT activities and TCho and TG concentrations were assessed in 30, 28, 27 and 15 dogs, respectively. Thrombocytosis (n = 30/43; 69.8%; $P = 0.0002$), elevated ALT (n = 41/44; 93.2%; $P < 0.0001$), elevated ALP (n = 42/44; 95.5%; $P = 0.0034$), and hypercalcemia (n = 13/32; 40.6%; $P = 0.0042$) were significantly associated with HCC.

Table 7. Breed distribution and statistics for dogs in the HCC group.

Breed	**n**	**Total n**	**OR**	**95% CI**	***P* value**
Welsh Corgi	7	224	3.79	1.67-8.60	0.0014*
Beagle	5	112	5.34	2.06-13.81	0.0006*
Shih Tzu	5	211	2.71	1.06-6.95	0.0378
Chihuahua	5	373	1.46	0.57-3.73	0.4274
Miniature Dachshund	4	852	0.44	0.16-1.23	0.1165
Yorkshire Terrier	4	163	2.77	0.98-7.84	0.0548
Toy Poodle	4	338	1.27	0.45-3.56	0.6546
Mongrel	4	342	1.25	0.44-3.51	0.6722
Pug	1	76	1.39	0.19-10.24	0.7455
Shiba Inu	1	179	0.57	0.08-4.18	0.5829
Boston Terrier	1	37	2.92	0.39-21.82	0.2953
Golden Retriever	1	94	1.12	0.15-8.20	0.9128
Miniature Schnauzer	1	167	0.62	0.08-4.50	0.6330
Pomeranian	1	125	0.83	0.11-6.09	0.8568

OR, odds ratio; 95% CI, 95% confidence interval.

**P* values < 0.0036 were statistically significant by Bonferroni correction.

Table 8. Concurrent diseases in the HCC group.

Category	**n**
Endocrinopathy/metabolic	
Hypothyroidism	2
Hyperadrenocorticism	10
Diabetes mellitus	1
Thyroid carcinoma	1
Hepatic/pancreatic	
Nodular hyperplasia	3
Gallbladder mucocele	1
Cardiovascular	
Myxomatous mitral valve degeneration	2
Heart-base tumor	1
Gastrointestinal	
Tooth root abscess	1
Leiomyoma of the ileum	1
Urinary	
Membranous glomerulonephritis	1
Chronic kidney disease	1
Bladder calculi	1
Neurological	
Idiopathic epilepsy	1
Cauda equina syndrome	1
Meningioma	1
Others	4
Total	33

Table 9. Univariate and multivariate logistic regression analysis of factors associated with HCC.

Variable	**Unadjusted OR (95% CI)**	***P* value**	**Adjusted OR (95% CI)**	***P* value**
Age	1.25 (1.13-1.38)	<0.0001*	1.20 (1.07-1.33)	0.0005*
Breed				
Welsh Corgis	3.79 (1.67-8.60)	0.0014*	3.68 (1.56-8.67)	0.0029*
Beagles	5.34 (2.06-13.81)	0.0006*	4.33 (1.58-11.90)	0.0044*
Shih Tzus	2.71 (1.06-6.95)	0.0378*	2.61 (0.98-6.99)	0.0556
Male sex	1.36 (0.74-2.51)	0.3186	1.47 (0.79-2.73)	0.2189
Concurrent disorder				
Hyperadrenocorticism	6.92 (3.37-14.22)	<0.0001*	4.13 (1.95-8.76)	0.0002*
Hypothyroidism	1.29 (0.31-5.36)	0.7302	0.82 (0.19-3.51)	0.7854
Diabetes mellitus	2.06 (0.28-15.23)	0.4799	1.69 (0.21-13.43)	0.6209

OR, odds ratio; 95% CI, 95% confidence interval.

**P* values < 0.05 were statistically significant.

Table 10. Hematologic and serum biochemical test results in the HCC and control groups.

Parameter	Reference range	HCC dogs (n = 44)			Control dogs (n = 44)			*P* value
		n	Median (range)	Abnormal (%)	n	Median (range)	Abnormal (%)	
Hematologic findings								
WBC count (×10³ cells/μL)	6.0-17.0	43	9.8 (5.4-23.5)	9.3	43	11.1 (4.8-47.9)	18.6	0.2072
HCT (%)	37.0-55.0	43	41.4 (20.6-57.7)	27.9	44	42.9 (14.8-54.5)	20.5	0.4607
PLT count (×10³ cells/μL)	20.0-50.0	43	57.5 (16.5-116)	69.8	43	40.3 (7.7-75.6)	25.6	0.0002*
Serum biochemical findings								
TP (g/dL)	5.0-7.2	44	7.3 (5.6-9.2)	0	44	6.9 (4.0-9.8)	4.6	0.1055
Alb (g/dL)	2.6-4.0	42	3.3 (2.1-4.8)	4.8	43	3.2 (1.2-3.9)	16.3	0.1561
Glu (mg/dL)	75-128	41	103 (38-168)	2.4	43	110 (72-226)	2.3	0.9717
ALT (IU/L)	17-78	44	314.5 (64-1001)	93.2	44	73 (17-1001)	45.5	<0.0001*
ALP (IU/L)	47-254	44	2551 (179-3591)	95.5	44	477 (72-3501)	70.5	0.0034*
AST (IU/L)	17-44	30	38.5 (17-369)	43.3	20	33.5 (12-848)	25.0	0.2370
GGT (IU/L)	5-14	28	13 (1-1076)	42.9	18	11 (0-1201)	38.9	1.0000
T-bil (mg/dL)	0.1-0.5	42	0.2 (0.1-0.5)	0	41	0.1 (0.1-10.1)	7.3	0.1160
tCa (mg/dL)	9.3-12.1	32	11.6 (8.9-13.6)	40.6	39	10.8 (6.9-13.2)	7.7	0.0042*
TCho (mg/dL)	111-312	27	227 (105-451)	44.4	21	258 (107-451)	33.3	0.5553
TG (mg/dL)	30-133	15	101 (58-354)	26.7	9	0.6 (47-501)	22.2	1.0000

Alb, albumin; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; Glc, glucose; HCT, hematocrit; PLT, platelet; T-bil, total bilirubin; tCa, total calcium; TCho, total cholesterol; TG, triglyceride; TP, total protein; WBC, white blood cell.

* *P* values < 0.05 were statistically significant.

4. DISCUSSION

This study investigated the prevalence, risk factors and clinical characteristics associated with HCC in dogs. Results of the present study revealed a higher prevalence of HCC than that observed in a previous study[1] and confirmed the risk of HCC development in older dogs, as reported in previous studies.[1,2] In addition, this study reported for the first time a breed predisposition for HCC in Welsh Corgis and Beagles and an association between HCC and hyperadrenocorticism. This study also found a significant association between dogs with HCC and thrombocytosis, elevated ALT and ALP and hypercalcemia. However, in contrast to the results of previous studies[1,2], there was no sex predisposition for HCC in this study.

In this study, the prevalence of HCC was higher than in a previous report, in which HCC was observed in 0.46% of dogs at necropsy.[1] This discrepancy might be due to recent advances in diagnostic technology and/or to differences in denominator populations. However, the results of the present study supported the results of previous studies that reported increased risk of HCC in dogs >10 years old.[1,2]

Interestingly, this study found an increased risk of HCC in Welsh Corgis and Beagles with a power of 75.3% and 76.9%, respectively. This result is inconsistent with previous studies reporting an overrepresentation of HCC in Miniature Schnauzers[18] and Shih Tzus.[19] Although Shih Tzus were predisposed to HCC in univariate analysis, the power statistic for Shih Tzus was only 46.6%. In addition, multivariate analysis confirmed that Shih Tzus were not predisposed to HCC. According to this analysis, there is low possibility that Shih Tzus are a predisposed breed in this study. However, a predisposition of Shih Tzus to HCC cannot be excluded based on underpowered statistics. Thus, further studies with a large number of Shih Tzus are needed to confirm the possibility that Shih Tzus are predisposed to HCC. Differences in breed predisposition among studies might also occur due to regional differences. Moreover,

it is possible that there are genetic differences in Welsh Corgis and Beagles from the area where this study was performed compared to those in other studies.

In humans, HCC is associated with chronic liver diseases, such as NASH. Previous studies have indicated an association between NASH and a metabolic syndrome characterized by lipid accumulation in hepatocytes.[43,44] Lipid accumulation leads to mitochondrial dysfunction, which results in oxidative stress in hepatocytes and can lead to the development of HCC.[50-52] In dogs, VH is a common hepatic disorder that has histopathologic characteristics similar to NASH in humans, although the pathophysiology of both disorders is different because VH is mostly associated with glycogen accumulation secondary to endogenous or exogenous glucocorticoid excess. However, NASH may be a form of VH since a previous study reported that VH in dogs also leads to mitochondrial dysfunction in hepatocytes, which is similar to the effects of NASH in humans.[22] Therefore, the association of HCC with NASH in humans may be similar to the association with VH in dogs. Thus, VH may contribute to the development of HCC in dogs.

A previous study in Scottish Terriers suggested that VH can cause hepatic remodelling and may progress to degenerative VH with the formation of regenerative foci. This transition may exhibit dysplastic characteristics and precede the development of HCC, as reported in human and experimental animal models.[20] However, other dog breeds can also develop degenerative VH, as reported in a previous study where an association between VH and neoplasia was suggested.[53] This indicates that it is possible that VH secondary to hyperadrenocorticism might play a role in the pathogenesis of HCC. Therefore, HCC should be considered when liver pathology is diagnosed in dogs with hyperadrenocorticism. However, the association between HCC and hyperadrenocorticism is in the present study is inconsistent with a recent report of disease associations in dogs with hyperadrenocorticism.[54] Differences in associated comorbidities might be due to difference between study designs. The present

study used the same period of disease occurrence as a condition for both HCC dogs and the reference population, in contrast to the previous report. Thus, it is possible that the association between HCC and hyperadrenocorticism could be present within the same period rather than at the same time point (i.e., death) since massive HCC can be treated by surgical resection before death.

This study did not find an association between HCC and hypothyroidism or diabetes mellitus, although, these two diseases are chronic disorders and can cause VH.[55] However, such associations cannot be certainly excluded due to the small number of HCC dogs with those two diseases. Thus, further studies are needed to investigate a large-scale HCC population to confirm the results of this study and determine whether there are any differences in the pathophysiology of VH in dogs with lipid and glycogen accumulation.

Although clinicopathologic features are usually nonspecific[27,28], thrombocytosis and hypercalcemia were overrepresented in the dogs with HCC examined here, which is similar to the results of previous reports.[2,18] The causes of HCC-related thrombocytosis and hypercalcemia in dogs are still unclear. These conditions may result from paraneoplastic syndrome, as observed in human HCC.[32-34] However, for hypercalcemia, the present study only evaluated the tCa concentration. Therefore, further investigation is needed to evaluate the ionized calcium concentration to confirm the presentation of hypercalcemia in dogs with HCC and determine whether these two conditions are paraneoplastic phenomena. Moreover, ALT and ALP levels were frequently increased in this study, supporting the results of previous studies, which reported that dogs with HCC typically present with high serum liver enzyme.[2,18] However, this observation is not specific for liver tumors.

This study had several limitations. Firstly, there was a small number of dogs with HCC, which may limit the ability to demonstrate an association in some breeds and with hypothyroidism or diabetes mellitus. Secondly, the association between HCC and long-term

steroid use, or the physiological effects of exogenous glucocorticoids on HCC development cannot be investigated in this study, due to the small number of HCC dogs with long-term glucocorticoid administration and the difficulty of collecting the history of long-term steroid use in the reference population because of the retrospective nature of this study. Thus, the possibility of HCC development associated with excess exogenous glucocorticoids remains unknown. In addition, due to the retrospective study design, clinicopathologic findings were not established for all dogs. Missing data may also have affected the results. Concurrent disorders occurring within 6 months of HCC presentation may not necessarily have been related to HCC, although this period provided adequate time for examining diseases suspected at the time of HCC diagnosis. There is also a possibility of false-positive diagnosis of hyperadrenocorticism in dogs with HCC. To minimize this limitation, stricter diagnostic criteria were used for hyperadrenocorticism, including only HCC dogs presenting with common clinical signs of hyperadrenocorticism other than abdominal distension and hepatomegaly in combination with positive endocrine tests. Finally, this retrospective study cannot confirm the role of hyperadrenocorticism in the pathogenesis of HCC development. Therefore, a prospective study with a large-scale population should be conducted to define any associations between HCC and hyperadrenocorticism or other comorbidities.

In conclusion, there was increased risk of HCC development with age, and Welsh Corgis and Beagles were predisposed to HCC. In addition, a significant association between HCC and hyperadrenocorticism was observed, suggesting that hyperadrenocorticism might be a predisposing factor for HCC development.

5. SUMMARY

In this chapter, the prevalence and potential risk factors associated with HCC in dogs were investigated. This retrospective study revealed a higher prevalence of HCC, presenting 0.96% than that observed in a previous study. The results confirmed the risk of the development of HCC in older dogs; however, there was no sex predisposition for HCC presented in this study. Clinicopathologic findings also found a significant presentation of thrombocytosis, high serum activities of ALT and ALP, and hypercalcemia in dogs with HCC. Additionally, the results suggested that Welsh Corgis and Beagles are breeds with a predisposition for HCC and that hyperadrenocorticism might be a potential risk factor.

GENERAL CONCLUSION

The goal of this study was to investigate the clinical utility of current diagnostic methods in distinguishing pathologic varieties of FLLs, and in order to gain new insight into the potential factors associated with HCC in dogs. The findings of the present study indicate that current diagnostic modalities, which is B-mode US, could predict pathologic varieties of FLLs including benign and malignant lesions via FLL appearances. Furthermore, the results of this study suggest a novel information for epidemiological features, including clinical features and risk factors of HCC in dogs.

In chapter 1, the clinical relevance of clinical and US data has been determined for the prediction of liver malignancy in dogs. Medical records and US images from dogs with FLL that underwent abdominal US and histopathologic examination following surgery or liver biopsy at HUVTH between 2013 and 2018 were retrospectively reviewed. The results of univariate analyses showed that several US features and PLT count were significantly associated with liver malignancy. Multivariate analysis revealed thrombocytosis, lesion size of 4.1 cm or greater, and heterogeneous echotexture of FLLs were independent predictors for differentiating benign and malignant liver lesions, suggesting that a combination of clinical data and US findings of FLLs could predict liver malignancy in dogs.

In chapter 2, the prevalence and potential risk factors associated with HCC in dogs have been investigated. Forty-four dogs with HCC presented to HUVTH from 2013 to 2017 were retrospectively reviewed. To examine the breed, age, sex predispositions or possible related factors for HCC including concurrent disorders, all dogs that came to the HUVTH during the study period were used as the reference population. Clinical characteristics of HCC were determined using propensity score matching analysis. As a result, the prevalence of HCC diagnosis was 0.96%. Multivariate analysis indicated an increased risk of HCC development

with age in dogs and showed that Welsh Corgis and Beagles are breeds with a predisposition for HCC. Twenty-seven of 44 dogs with HCC had at least one concurrent disorder. The most common concurrent disorder was hyperadrenocorticism. Propensity score matching analysis revealed that thrombocytosis, increased ALT, increased ALP, and hypercalcemia were significantly associated with HCC. These results suggested that Welsh Corgis and Beagles are breeds with a predisposition for HCC and that hyperadrenocorticism might be a potential risk factor.

In order to confirm the clinical utility of the combination of both thrombocytosis and B-mode US features for liver malignancy detection, as well as the potential risk factor associated with HCC, further investigations should include a large-scale of dog population with multiple institution. Furthermore, to clarify the underlying pathogenesis between hyperadrenocorticism and HCC, additional research by collecting liver tissues is needed for in-depth evaluation in the future which may support the hypothesis of this study that VH may be a form of NASH in humans and possibly precede to the development of HCC.

In conclusion, through this study the clinical utility of current diagnostic methods, including B-mode US and clinical data, for distinguishing pathologic varieties of FLLs was investigated. In addition, the potential factors associated dogs with HCC were clarified for gaining new insight into clinical aspect of HCC. The use of current diagnostic methods which are a combination of clinical data and US findings of FLLs could predict liver malignancy in dogs. On the other hand, regarding the clinical aspect of HCC in dogs, this study showed Welsh Corgis and Beagles are breeds with a predisposition for HCC and that hyperadrenocorticism might be a potential risk factor. The results of this study could provide the useful information and fulfill the aspect of clinical diagnosis of FLLs in dogs for clinicians in clinical application in the future.

JAPANESE SUMMARY（要旨）

The study on diagnosis and clinical aspects of focal liver lesion in dogs

（犬の肝局所性病変の診断ならびに臨床的研究）

小動物臨床において、犬の肝臓腫瘤に遭遇する機会は比較的多い。これらの犬の多くが、肝臓腫瘤に関連する徴候を主訴に動物病院を受診する。犬の肝臓腫瘤には良性病変と悪性病変が含まれるため、手術適応を判断するための暫定診断は臨床的に重要である。しかし、ゴールドスタンダードとして用いられている肝生検は侵襲的な検査であり、結果として生命を脅かす合併症を引き起こす可能性がある。そのため、肝臓腫瘤の病理学的な特徴を予測するための非侵襲的診断法が依然として必要とされている。

一般的に、肝臓腫瘤を臨床徴候、血液検査および腹部 X 線検査によって診断することは困難である。一方、近年広く用いられるようになった腹部超音波検査では容易に検出可能であるため、肝臓腫瘤が偶発的に発見される動物の数は増加している。したがって、腹部超音波検査の所見に基づいた肝臓腫瘤の特徴が明らかになれば、悪性病変と良性病変を区別するための貴重な情報となるものと考えられる。

Ｂモード超音波検査は、肝臓を探査するために臨床現場で一般的に使用されている診断方法である。しかしながら、肝臓腫瘤の良悪性鑑別においては診断的価値のある情報を提供することは困難であると考えられてきた。一方、最近の研究では、B モード超音波検査所見と悪性腫瘍の関連が示唆されている。さらに、シグナルメント、臨床徴候および臨床病理学所見などの臨床データだけでは、肝臓腫瘤の

原因を特定するのに不十分である。しかしながら、肝臓腫瘤における臨床データと超音波所見を組み合わせることで、良性病変と悪性病変を予測できる可能性がある。

加えて、犬において最も一般的な原発性肝臓腫瘍である肝細胞癌の疫学的特徴に関する情報もほとんど明らかになっていない。

したがって、上記の背景を考慮し、私は犬の限局性肝臓病変の診断および臨床的特徴に関する研究を行った。第1章では、2013年から2018年の間に北大動物医療センターを訪れた83例の犬において、肝臓の悪性腫瘍を予測する臨床所見および超音波検査所見について検討した。その結果、血小板増加症、4.1cm 以上の病変サイズおよび肝臓腫瘤の不均一なエコー源性が、良性病変と悪性病変を区別するための独立した予測因子であり、肝臓腫瘤の臨床データと超音波検査所見を組み合わせることで、肝臓の悪性病変を予測できることが示唆された。

第2章では、2013年から2017年の間に北大動物医療センターで診断された44例の肝細胞癌症例から、犬の肝細胞癌の有病率および危険因子を調査した。その結果、ウェルシュ・コーギーとビーグルは肝細胞癌の好発犬種であることが明らかになった。さらに、肝細胞癌と副腎皮質機能亢進症との間に有意な関連が認められ、副腎皮質機能亢進症が肝細胞癌の危険因子となる可能性が示唆された。

結論として、本研究ではＢモード超音波検査所見と臨床データの組み合わせに関して肝臓腫瘤の良悪性鑑別における有用性を検討した。その結果、臨床データ、超音波検査所見を組み合わせることで、犬の肝臓悪性腫瘍を予測することができると考えられた。加えて、肝細胞癌の疫学的特徴を調査し、ウェルシュ・コーギーとビーグルが肝細胞癌の好発品種であり、副腎皮質機能亢進が肝細胞癌の危険因

子である可能性を示した。これらすべての結果は犬の肝臓腫瘤における臨床診断において有用な情報となる。

REFERENCES

1. Patnaik, A. K., Hurvitz, A. I., Lieberman, P. H. and Johnson, G. F. 1981.Canine Hepatocellular Carcinoma. *Vet. Pathol*., **18**: 427–438.
2. Patnaik, A. K., Hurvitz, A. I. and Lieberman, P. H. 1980. Canine Hepatic Neoplasms: A Clinicopathologic Study. *Vet. Pathol*., **17**: 553–564.
3. Liptak, J. M. 2013. Hepatobiliary tumors. pp. 405–412. In: Withrow and MacEwen's Small Animal Clinical Oncology, 5th ed. (Withrow, S. J., Vail, D. M. and Page, R. L. eds.), Elsevier, St. Louis.
4. Rothuizen, J. and Twedt, D. C. 2009. Liver biopsy techniques. *Vet. Clin. North Am. Small Anim. Pract*., **39**: 469–480.
5. Bigge, L. A., Brown, D. J. and Penninck, D. G. 2001. Correlation between coagulation profile findings and bleeding complications after ultrasound-guided biopsies: 434 cases (1993–1996). *J. Am. Anim. Hosp. Assoc*., **37**: 228–233.
6. Léveillé, R., Partington, B. P., Biller, D. S. and Miyabayashi, T. 1993. Complications after ultrasound-guided biopsy of abdominal structures in dogs and cats: 246 cases (1984–1991). *J. Am. Vet. Med. Assoc*., **203**: 413–415.
7. Kanemoto H, Ohno K, Nakashima K, Takahashi, M., Fujino, Y., Nishimura, R. and Tsujimoto H. 2009. Characterization of canine focal liver lesions with contrast-enhanced ultrasound using a novel contrast agent—Sonazoid. *Vet. Radiol. Ultrasound*, **50**: 188–194.
8. Nakamura, K., Takagi, S., Sasaki, N., Bandula Kumara, W. R., Murakami, M., Ohta, H., Yamasaki, M. and Takiguchi, M. 2010. Contrast-enhanced ultrasonography for characterization of canine focal liver lesions. *Vet. Radiol. Ultrasound*, **51**: 79–85.
9. Biller, D. S. and Blackwelder, T. 1998. Hepatic ultrasonography: a valuable tool in small animals. *Vet. Med*., **93**: 646–653.

10. Stowater, J. L., Lamb, C. R. and Schelling, S. H. 1990. Ultrasonographic features of canine hepatic nodular hyperplasia. *Vet. Radiol. Ultrasound*, **31**: 268–272.
11. Cuccovillo, A. and Lamb, C. R. 2002. Cellular features of sonographic target lesions of the liver and spleen in 21 dogs and a cat. *Vet. Radiol. Ultrasound*, **43**: 275–278.
12. d'Anjou, M. A. and Penninck, D. 2015. Liver. pp. 183–238. In: Atlas of Small Animal Ultrasonography, 2nd ed. (Penninck, D. and d'Anjou, M. A. eds.), Blackwell, Ames.
13. Nyland, T. G., Larson, M. M. and Mattoon, J. S. 2015. Liver. pp. 332–399. In: Small Animal Diagnostic Ultrasound, 3rd ed. (Mattoon, J. S. and Nyland, T. G. eds.), Elsevier, St. Louis.
14. Saunders, H. M. 1998. Ultrasonography of abdominal cavitary parenchymal lesions. *Vet. Clin. North Am. Small Anim. Pract.*, **28**: 755–775.
15. Whiteley, M. B., Feeney, D. A., Whiteley, L. O. and Hardy, R. M. 1989. Ultrasonographic appearance of primary and metastatic canine hepatic tumors: a review of 48 cases. *J. Ultrasound Med.*, **8**: 621–630.
16. Griebie, E. R., David, F. H., Ober, C. P., Feeney, D. A., Anderson, K. L., Wuenschmann, A. and Jessen, C. R. 2017. Evaluation of canine hepatic masses by use of triphasic computed tomography and B-mode, color flow, power, and pulsed-wave Doppler ultrasonography and correlation with histopathologic classification. *Am. J. Vet. Res.*, **78**: 1273–1283.
17. Gumerlock, P. H., Kraegel, S. A. and Madewell, B. R. 1992. Detection of mammalian and avian hepadenovirus by the polymerase chain reaction. *Vet. Microbiol.*, **32**: 273–280.
18. Liptak, J. M., Dernell, W. S., Monnet, E., Powers, B. E., Bachand, A. M., Kenney, J. G. and Withrow, S. J. 2004. Massive hepatocellular carcinoma in dogs: 48 cases (1992–2002). *J. Am. Vet. Med. Assoc.*, **225**: 1225–1230.

19. Hirose, N., Uchida, K., Kanemoto, H., Ohno, K., Chambers, J. K. and Nakayama, H. 2014. A Retrospective Histopathological Survey on Canine and Feline Liver Diseases at the University of Tokyo between 2006 and 2012. *J. Vet. Med. Sci.*, **76**: 1015–1020.
20. Cortright, C. C., Center, S. A., Randolph, J. F., McDonough, S. P., Fecteau, K. A., Warner, K. L., Chiapella, A. M., Pierce, R. L., Graham, A. H., Wall, L. J., Heidgerd, J. H., Degen, M. A., Lucia, P. A. and Erb, H. N. 2014. Clinical features of progressive vacuolar hepatopathy in Scottish Terriers with and without hepatocellular carcinoma: 114 cases (1980-2013). *J. Am. Vet. Med. Assoc.*, **245**: 797–808.
21. Peyron, C., Chevallier, M., Lecoindre, P., Guerret, S. and Pagnon, A. 2014. Clinical, blood biochemical and hepatic histological data in 49 French Scottish Terriers dogs according to their plasma ALP activity, hepatic vacuolation and the presence or absence of hepatocellular carcinoma. *Revue Méd. Vét.*, **165**: 245–251.
22. Peyron, C., Lecoindre, P., Chevallier, M., Guerret, S. and Pagnon, A. 2015. Vacuolar hepatopathy in 43 French Scottish Terriers: a morphological study. *Revue Méd. Vét.*, **166**: 176–184.
23. Guillot, M., d'Anjou, M., Alexander, K., Bédard, C., Desnoyers, M., Beauregard, G. and Del Castillo, J. R. 2009. Can sonographic findings predict the results of liver aspirates in dogs with suspected liver disease? *Vet. Radiol. Ultrasound*, **50**: 513–518.
24. Murakami, T., Feeney, D. A. and Bahr, K. L. 2012. Analysis of clinical and ultrasonographic data by use of logistic regression models for prediction of malignant versus benign causes of ultrasonographically detected focal liver lesions in dogs. *Am. J. Vet. Res.*, **73**: 821–829.
25. Warren-Smith, C. M. R., Andrew, S., Mantis, P. and Lamb, C. R. 2012. Lack of associations between ultrasonographic appearance of parenchymal lesions of the canine liver and histological diagnosis. *J. Small Anim. Pract.*, **53**: 168–173.

26. Badea, R. and Ioanitescu, S. 2012. Ultrasound imaging of liver tumors – current clinical applications. pp. 75–102. In: Liver Tumors, ed. (Julianov, A. ed.), InTech, Croatia. http://www.intechopen.com/books/liver-tumors/ultrasound-imaging-of-liver-tumors-current-clinical-applications. (assessed 6 September 2017).
27. Bexfield, N. 2017. Neoplasms of the liver. pp. 4065–4074. In: Textbook of Veterinary Internal Medicine, 8th ed. (Ettinger, S. J., Feldman, E. C. and Cote, E. eds.), Elsevier, St. Louis.
28. Selmic, L. E. 2017. Hepatobiliary Neoplasia. *Vet. Clin. North Am. Small Anim. Pract.*, **47**: 725–735.
29. Neel, J. A., Snyder, L. and Grindem, C. B. Thrombocytosis: A retrospective study of 165 dogs. 2012. *Vet. Clin. Pathol.*, **41**: 216–222.
30. Athanasiou, L. V., Polizopoulou, Z. S., Papavasileiou, E. G., Mpairamoglou, E. L., Kantere, M. C. and Rousou, X. A. 2017. Magnitude of reactive thrombocytosis and associated clinical conditions in dogs. *Vet. Rec.*, **181**: 1–4.
31. Woolcock, A. D., Keenan, A., Cheung, C., Christian, J. A. and Moore, G. E. 2017. Thrombocytosis in 715 Dogs (2011–2015). *J. Vet. Intern. Med.*, **31**: 1691–1699.
32. Luo, J. C., Hwang, S. J., Wu, J. C., Li, C. P., Hsiao, L. T., Lai, C. R., Chiang, J. H., Lui, W. Y., Chang, F. Y. and Lee, S. D. 1999. Paraneoplastic syndromes in patients with hepatocellular carcinoma in Taiwan. *Cancer*, **86**: 799–804.
33. Hwang, S. J., Luo, J. C., Li, C. P., Chu, C. W., Wu, J. C., Lai, C. R., Chiang, J. H., Chau, G. Y., Lui, W. Y., Lee, C. C., Chang, F. Y. and Lee, S. D. 2004. Thrombocytosis: a paraneoplastic syndrome in patients with hepatocellular carcinoma. *World J. Gastroenterol.*, **10**: 2472–2477.
34. Chang, P. E., Ong, W. C., Lui, H. F. and Tan, C. K. 2013. Epidemiology and prognosis of paraneoplastic syndromes in hepatocellular carcinoma. *ISRN Oncology*, **2013**: 684026.

35. Suzuki, A., Takahashi, T., Nakamura, K., Tsuyuoka, R., Okuno, Y., Enomoto, T., Fukumoto, M. and Imura H. 1992. Thrombocytosis in patients with tumors producing colony-stimulating factor. *Blood*, **80**: 2052–2059.

36. Sasaki, Y., Takahashi, T., Miyazaki, H., Matsumoto, A., Kato, T., Nakamura, K., Iho, S., Okuno, Y. and Nakao, K. 1999. Production of thrombopoietin by human carcinomas and its novel isoforms. *Blood*, **94**: 1952–1960.

37. Bihari, C., Rastogi, A., Shasthry, S. M., Bajpai, M., Bhadoria, A. S., Rajesh, S., Mukund, A., Kumar, A. and Sarin, S. K. 2016. Platelets contribute to growth and metastasis in hepatocellular carcinoma. *APMIS*., **124**: 777–786.

38. Hassan, M. M., Kaseb, A., Li, D., Patt, Y. Z., Vauthey, J. N., Thomas, M. B., Curley, S. A., Spitz, M. R., Sherman, S. I., Abdalla, E. K., Davila, M., Lozano, R. D., Hassan, D. M., Chan, W., Brown, T. D. and Abbruzzese, J. L. 2009. Association between hypothyroidism and hepatocellular carcinoma: a case-control study in the United States. *Hepatology*, **49**: 1563–1570.

39. Wang, Y. G., Wang, P., Wang, B., Fu, Z. J., Zhao, W. J. and Yan, S. L. 2014. Diabetes mellitus and poorer prognosis in hepatocellular carcinoma: a systematic review and meta-analysis. *PLoS One*, **9**: e95485.

40. Banal, K. A., Paz-Pacheco, E. and de Villa, V., 2017. Diabetes mellitus and prediabetes in patients with hepatocellular carcinoma in a tertiary Philippine hospital. *JAFES*., **32**: 32–37.

41. Liangpunsakul, S. and Chalasani, N. 2003. Is hypothyroidism a risk factor for nonalcoholic steatohepatitis? *J. Clin. Gastroenterol*., **37**: 340–343.

42. El-Serag, H. B., Hampel, H. and Javadi, F. 2006. The association between diabetes and hepatocellular carcinoma: a systematic review of epidemiologic evidence. *Clin. Gastroenterol. Hepatol*., **4**: 369–380.

43. Fingas, C. D., Best, J., Sowa, J. P. and Canbay, A. 2016. Epidemiology of nonalcoholic steatohepatitis and hepatocellular carcinoma. *Clin*. Liver *Dis*. (Hoboken)., **8**: 119–122.

44. Cholankeril, G., Patel, R., Khurana, S. and Satapathy, S. K. 2017. Hepatocellular carcinoma in non-alcoholic steatohepatitis: current knowledge and implications for management. *World J. Hepatol*., **9**: 533–543.

45. Takaki, A., Kawai, D. and Yamamoto, K. 2013. Multiple hits, including oxidative stress, as pathogenesis and treatment target in non-alcoholic steatohepatitis (NASH). *Int. J. Mol. Sci*., **14**: 20704–20728.

46. Cullen, J. M. 2009. Summary of the World Small Animal Veterinary Association standardization committee guide to classification of liver disease in dogs and cats. *Vet. Clin. North Am. Small Anim. Pract*., **39**: 395–418.

47. Reusch, C. E., 2015. Glucocorticoid therapy. pp. 555–577. In: Canine and Feline Endocrinology, 4th ed. (Feldman, E. C., Nelson, R. W., Reusch, C. and Scott-Moncrieff, J. C. eds.), Elsevier, St. Louis.

48. Mesich, M. L. L., Mayhew, P. D., Paek, M., Holt, D. E. and Brown, D. C. 2009. Gall bladder mucoceles and their association with endocrinopathies in dogs: a retrospective case-control study. *J.* Small *Anim. Pract*., **50**: 630–635.

49. Behrend, E. N., Kooistra, H. S., Nelson, R., Reusch, C. E., and Scott-Moncrieff, J. C. 2013. Diagnosis of spontaneous canine hyperadrenocorticism: 2012 ACVIM consensus statement (small animal). *J. Vet. Intern. Med*., **27**: 1292–1304.

50. Paschos, P. and Paletas, K. 2009. Non alcoholic fatty liver disease and metabolic syndrome. *Hippokratia*, **13**: 9–19.

51. Vanni, E., Bugianesi, E., Kotronen, A., De Minicis, S., Yki-Järvinen, H. and Svegliati-Baroni, G. 2010. From the metabolic syndrome to NAFLD or vice versa? *Dig. Liver Dis*., **42**: 320–330.

52. Eshraghian, A. and Jahromi, A. H. 2014. Non-alcoholic fatty liver disease and thyroid dysfunction: a systematic review. *World J. Gastroenterol*., **20**: 8102–8109.
53. Sepesy, L. M., Center, S. A., Randolph, J. F., Warner, K. L. and Erb, H. N. 2006. Vacuolar hepatopathy in dogs: 336 cases (1993-2005). *J. Am. Vet. Med. Assoc*., **229**: 246–252.
54. Hoffman, J. M., Lourenço, B. N., Promislow, D. E. L. and Creevy, K. E. 2018. Canine hyperadrenocorticism associations with significant, selected comorbidities and mortality within North American veterinary teaching hospitals. *J. Small Anim. Pract*., **59**: 681–690.
55. Watson, P. J. 2017. Metabolic diseases of the liver. pp. 4037–4051. In: Textbook of Veterinary Internal Medicine, 8th ed. (Ettinger, S. J., Feldman, E. C. and Cote, E. eds.), Elsevier, St. Louis.

ACKNOWLEDGEMENTS

The completion of this thesis would not have been possible without countless people who have contributed to scientific contents, supported and encouraged me.

First of all, I would like to express my sincere gratitude to the person who made it all possible, my supervisor, Dr. Mitsuyoshi Takiguchi (Graduate School of Veterinary Medicine, Hokkaido University) for giving me the opportunities to achieve my goals and aspirations, and for his continuous support, guidance, invaluable advice, supervision and attention given to me throughout my study.

I also wish to thank the rest of my co-advisors and thesis committee; Drs. Takashi Kimura (Graduate School of Veterinary Medicine, Hokkaido University), Hiroshi Ohta (Graduate School of Veterinary Medicine, Hokkaido University), Kensuke Nakamura (Organization for Promotion of Tenure Track, University of Miyazaki), Satoshi Takagi (Department of Veterinary Medicine, Azabu University) and Keitaro Morishita (Graduate School of Veterinary Medicine, Hokkaido University) for their insightful comments and encouragements into making this study a better one. I am also exceptionally grateful to my mentor Dr. Hiroshi Ohta (Graduate School of Veterinary Medicine, Hokkaido University) for his time and effort in critical evaluating my research, papers and presentations. Without him, my work would not have been improved to its current state.

My sincere thanks also go to Drs. Kenji Hosoya (Graduate School of Veterinary Medicine, Hokkaido University), Noboru Sasaki (Graduate School of Veterinary Medicine, Hokkaido University), Tatsuyuki Osuga (Graduate School of Veterinary Medicine, Hokkaido University) and Genya Shimbo (Graduate School of Veterinary Medicine, Hokkaido University) for their constant support and assistance. I also wish to thank my wonderful senior;

Dr. Khoirun Nisa, my colleague; Dr. Angkhana Dermlim and my fellow comrades; Drs. Kazuyoshi Sasaoka, Noriyuki Nagata, Masahiro Tamura for their great help and support.

In addition, I am also thankful to all members of the Laboratory of Veterinary Internal Medicine of Hokkaido University and all staffs of Hokkaido University Veterinary Teaching Hospital for their kind support during the study period and for sharing every unforgettable moment and being my family here in Sapporo.

Last but not least, I would like to thank my friends and family for all their love, moral support and warm encouragement throughout my years of study and through the process of researching and writing this thesis.

This accomplishment would not have been possible without all these people.