

## Diagnostic utility of basal serum T4 and TSH concentrations in dogs with hypothyroidism confirmed by TSH stimulation test

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### *Abstract*

The objective of this study was to evaluate the diagnostic performance of a basal serum thyroxine (T4) and thyrotropin (TSH) combination test for the diagnosis of canine hypothyroidism in clinical practice. It was hypothesized that the usefulness of basal T4 and TSH combination test in dogs with suspected non-thyroidal illness syndrome would be different with that in previous studies. The data of this study were searched for dogs who underwent both TSH stimulation test and basal serum T4 and TSH combination test. Forty-four cases were classified into either of three groups (hypothyroid, intermediate, or euthyroid) according to the results of TSH stimulation test, and the basal T4 and TSH concentrations were compared among the three groups. The usefulness of the T4 and TSH combination test regarding the diagnosis made by the TSH stimulation test was evaluated. Laboratory findings were compared among the three groups. Seventeen cases were classified into the hypothyroid group, 11 into the intermediate group, and 16 into the euthyroid group. Of the 13 cases with low T4 and high TSH concentrations, only 7 cases were diagnosed with hypothyroidism and 3 cases were considered euthyroid. There were no significant differences in laboratory results among the three groups. The basal serum T4 and TSH combination test was useful to reject hypothyroidism but was not enough to confirm it. When dogs are suspected to have non-thyroidal illness syndrome, these test could lead to false positives. Although thyroid drug trial may be effective depending on the basal T4 and TSH combination test, TSH stimulation tests are encouraged to confirm canine hypothyroidism.

**Keywords:** canine, non-thyroidal illness syndrome, hypothyroidism, TSH stimulation test, basal T4 and TSH combination test

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Received February 11, 2021

Accepted July 1, 2021

<https://doi.org/10.14456/tjvm.2021.79>

## Introduction

Hypothyroidism is a common endocrine disorder in dogs (Dixon *et al.*, 1999; Milne and Hayes Jr, 1981; Panciera, 1994). It is mostly caused by lymphocytic thyroiditis or idiopathic atrophy (Lucke *et al.*, 1983). As thyroid hormones have many metabolic functions, the clinical signs of hypothyroidism are various and non-specific (Daminet *et al.*, 2007; David, 2001). Serum total thyroxine (T4), free T4, thyrotropin (TSH), and thyroglobulin autoantibody can be used for the diagnosis of canine hypothyroidism (Beale, 1991; Iversen *et al.*, 1998; Nachreiner *et al.*, 1998). Generally, low serum T4 level combined with a high serum TSH concentration is suggestive of hypothyroidism in veterinary medicine (Mooney, 2017). However, no single or combination hormone test has 100% diagnostic reliability (Boretti and Reusch, 2004; Dixon and Mooney, 1999; Kempainen and Behrend, 2001; Peterson *et al.*, 1997; Ramsey *et al.*, 1997). Non-thyroidal illness syndrome (NTIS), defined as an alteration of thyroid hormone metabolism due to a non-thyroidal disease, and certain drugs can affect serum T4 and TSH levels (Nishii *et al.*, 2019). Therefore, measurement of basal serum thyroid hormone levels cannot precisely distinguish between hypothyroidism and NTIS, making the diagnosis of hypothyroidism challenging (Pijnacker *et al.*, 2018).

There are other methods for the assessment of thyroid function that can overcome the abovementioned limitation. The TSH stimulation test is considered the gold standard for diagnosing canine hypothyroidism (Daminet *et al.*, 2007). This test is recommended for dogs whose diagnosis cannot be confirmed using clinical signs and basal hormone tests, and it is also useful in dogs with concurrent systemic illnesses or receiving drugs known to affect thyroid metabolism (Boretti *et al.*, 2006; Panciera, 1999). However, some dogs cannot be classified into either hypothyroid or euthyroid groups. Results of an intermediate group are difficult to interpret, and further or re-examination should be considered. Moreover, due to the high cost of recombinant human (rh) TSH, this test is not being routinely used as a diagnostic option in veterinary clinics (Pijnacker *et al.*, 2018). Although TSH stimulation test is the gold standard, the fact that the thyroid function of the intermediate group is difficult to assess is a limitation. Until now, only two studies have incorporated the intermediate group, and these studies were intended to evaluate the appropriate dose of rhTSH to use in the TSH stimulation test (Boretti *et al.*, 2006; Boretti *et al.*, 2009). In order to diagnose hypothyroidism more accurately, additional studies are needed to assess the characteristics of the intermediate group. Thyroid scintigraphy, which is based on radioactive pertechnetate uptake, is another useful option for the diagnosis of canine hypothyroidism. This method can differentiate between hypothyroid dogs and dogs with NTIS (Espineira *et al.*, 2007; Shiel *et al.*, 2012). However, scintigraphy is only available in a few referral hospitals that have a gamma camera and a radiation-isolation system (Pijnacker *et al.*, 2018).

Consequently, the measurement of basal serum T4 and TSH concentrations is the most cost effective and

widely available test in veterinary practice (Mooney, 2017). The sensitivity and specificity of basal T4 and TSH levels for the diagnosis of hypothyroidism are reported to be 89-100% and 73-82% and 58-87% and 82-100%, respectively (Boretti and Reusch, 2004; Dixon and Mooney, 1999; Kempainen and Behrend, 1998; Peterson *et al.*, 1997). Additionally, the sensitivity and specificity of the T4 and TSH combination test are reported to be 63-67% and 98-100%, respectively (Ferguson, 2007; McCann, 2015). However, these studies were published 6 to 23 years ago. Nowadays, the improvement in diagnostic test performance and the increased knowledge and experience regarding hypothyroidism may lead to different results.

The aim of the present study was to evaluate the diagnostic performance of basal serum T4 and TSH combination test for the diagnosis of canine hypothyroidism in clinical practice. It was hypothesized that the usefulness of T4 and TSH measurements in this study would be different with that in previous studies, and that laboratory findings would help to distinguish the intermediate group from the hypothyroid and euthyroid groups. The TSH stimulation test is more accurate for assessing thyroid function than is the measurement of basal thyroid hormone concentrations. Moreover, the TSH stimulation test is usually indicated to differentiate hypothyroidism from NTIS in dogs with low basal thyroid hormone concentrations (Scott-Moncrieff, 2015). Because the present study was undertaken to examine the utility of basal T4/TSH for the diagnosing hypothyroidism in dogs with hypothyroidism with/without a variety of concurrent diseases in clinical practice, we also used the TSH stimulation test to distinguish hypothyroid from non-hypothyroid dogs.

## Materials and Methods

The study was approved by the Institutional Animal Care and Use Committee of the Laboratory Animal Research Center of Chungbuk National University (approval No. CBNUA-1601-17-01).

**Case allocation and assays:** The data of this study were searched for dogs that underwent both TSH stimulation test and basal serum T4 and TSH combination test between April 2014 and November 2019. The inclusion criteria for dogs in the present study were the availability of concurrent measurements of serum basal T4, TSH, and post-TSH T4 concentrations. Serum T4 and TSH concentrations were measured by a chemiluminescent assay (Immulite 2000, Siemens Co., Munich, Germany). The reference interval levels for T4 was 1 to 4 µg/dL and for TSH, 0.05 to 0.42 ng/mL. Basal serum T4 and TSH concentrations were routinely measured for the diagnosis of hypothyroidism. The combination of reduced T4 and elevated TSH level led to a putative diagnosis of hypothyroidism (Mooney, 2017). However, even with low T4 and high TSH, dogs suspected of NTIS were tested with the TSH stimulation test. The TSH stimulation test was also performed in dogs suspected of NTIS with low T4 and normal TSH and normal T4 and elevated TSH. In

addition, some dogs were subjected to TSH stimulation tests to determine the cause of seizure. The exclusion criteria were incomplete or missing anamnestic data and levothyroxine treatment within 2 months preceding the thyroid function tests. Finally, forty-four cases (forty-two dogs; two dogs were tested twice with an in-between test interval) were included in this study. The medical records (signalment, laboratory findings, and concurrent diseases) from the enrolled cases were collected for data analysis. Complete blood count and blood chemistry analyses were conducted as part of the diagnostic tests, and all the owners were informed about the use of their dogs' laboratory findings for research purposes.

**TSH stimulation test:** rhTSH (Thyrogen; Zenzyme, Cambridge, MA, USA) was used for the TSH stimulation test. One vial of rhTSH (1.1 mg) was diluted in 1.2 mL of sterile water and distributed into eight plastic syringes with rubber caps. These syringes were stored at -20 °C, for a maximum of 12 weeks, until the assay was performed (Roover *et al.*, 2006; Daminet *et al.*, 2007). The frozen rhTSH was thawed at room temperature. During the test, 100 µg of diluted rhTSH was administered through the cephalic vein. Blood samples were collected from the jugular vein before and 6 hours after administration. Cases were classified based on the following criteria: dogs with a pre- and post-administration T4 concentration < 1.5 µg/dL were classified into the hypothyroid group, dogs with a post-administration T4 concentration > 2.5 µg/dL and at least 1.5 times higher than the pre-administration T4 concentration were classified into the euthyroid group, and dogs with a post-administration T4 concentration between 1.5 and 2.5 µg/dL or with post-administration T4 concentration of > 2.5 µg/dL but an increase of < 1.5 times compared to pre-administration T4 concentration were classified into the intermediate group (Scott-Moncrieff, 2015).

**Statistical analyses:** Data were analyzed using IBM SPSS Statistics Version 22 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism Version 6.01 (GraphPad Software, La Jolla, CA, USA). A value of  $P < 0.05$  was considered statistically significant. An analysis of variance and a Kruskal-Wallis test, with Post-hoc tests, using Tukey's and Dunn's multiple comparison tests, respectively were used to compare age, T4 and TSH concentrations, and laboratory results among the hypothyroid, intermediate, and euthyroid groups. After univariate analyses, variables with  $P < 0.20$  were included in the subsequent multivariate analyses (Bursac *et al.*, 2008; Hosmer *et al.*, 2013). Odds ratio (OR) and 95% confidence intervals (CI) of basal serum T4 concentration and other variables with  $P < 0.20$  after univariate analyses were estimated using forward conditional multiple logistic regression analyses (Bursac *et al.*, 2008; Hosmer *et al.*, 2013). Furthermore, the receiver operating characteristics (ROC) area under the curve (AUC) was used to assess the optimal cut-off value of basal serum T4 concentration for the diagnosis of hypothyroidism in dogs with and without specific concurrent diseases. This optimal cut-off value of basal serum T4 concentration was identified after multiple

logistic regression analyses to influence its diagnostic performance in hypothyroidism diagnosis.

## Results

Forty-four cases who underwent both basal T4 and TSH combination test and TSH stimulation test were included in this study. Dog breeds included 11 shih tzus, 9 Maltese, 4 toy poodles, 3 miniature schnauzers, 2 chihuahuas, 2 Pomeranians, and 1 each of Cocker spaniel, dachshund, golden retriever, Pekingese, and Yorkshire terrier, and 8 Mixed breed. They were classified as hypothyroid ( $n=17$ ), intermediate ( $n=11$ ), and euthyroid ( $n=16$ ) based on the results of the TSH stimulation test. Among all the dogs, concurrent diseases included chronic kidney disease (CKD), chronic valvular heart disease, hyperadrenocorticism (HAC), anemia, neurologic disease, dermatologic disease, and diabetes mellitus, and the consumed medications were phenobarbital and glucocorticoids (Table 1). In the intermediate and euthyroid groups, age ranged from 1 to 17 years old, whereas the minimum age was over 10 years old in the hypothyroid group. Statistically, cases in the euthyroid group were significantly younger ( $P = 0.0034$ ) than in those in the other two groups.

The range of basal T4 concentration was 0.23 to 0.97 µg/dL, 0.40 to 1.20 µg/dL, and 0.31 to 3.14 µg/dL in the hypothyroid, intermediate, and euthyroid groups, respectively (Fig. 1). The basal T4 concentration was significantly lower ( $P = 0.0012$ ) in the hypothyroid group compared with that of the euthyroid group. However, no significant difference in basal T4 concentration between the intermediate group and the hypothyroid and euthyroid groups was noted ( $P > 0.05$ ). The range of TSH concentration was 0.03 to 2.11 ng/mL, 0.15 to 0.69 ng/mL, and 0.06 to 0.87 ng/mL in the hypothyroid, intermediate, and euthyroid groups, respectively (Fig. 2). There were no significant differences in serum TSH concentration among the three groups ( $P > 0.05$ ).

The distribution of cases according to the pre-administration T4 concentration among the three groups is summarized in Table 2. When the basal T4 concentration was lower than 0.5 µg/dL, 66.6% of the cases were diagnosed with hypothyroidism. In the 0.5 - 1.0 µg/dL range, the representation of the three groups was similar, but there were more hypothyroid cases (40.9%) than intermediate (31.8%) and euthyroid (27.3%). When the basal T4 concentration was within the reference interval (1 to 4 µg/dL), no case was diagnosed with hypothyroidism. Two out of 10 cases were classified as intermediate, and the remaining 8 cases were classified as euthyroid. All cases with T4 concentrations higher than 1.5 µg/dL were classified as euthyroid.

When comparing the results of the T4 and TSH combination test and the TSH stimulation tests, 53.8% of cases with reduced T4 and elevated TSH concentrations were diagnosed as hypothyroid, 23.1% were diagnosed as intermediate, and 23.1% were diagnosed as euthyroid (Table 3). Similar results were found in cases with low T4 and normal TSH levels; 47.6% of cases were diagnosed as hypothyroid, 28.6% were diagnosed as intermediate, and 23.8% were

diagnosed as euthyroid. Only 50% of cases with reduced basal T4 concentration were determined to be in the hypothyroid group. No dogs were diagnosed with hypothyroidism in cases of normal T4 level.

Regarding the laboratory results, there were no significant differences among the three groups ( $P > 0.05$ ; Table 4).

After univariate analyses, the variables such as age, neurologic disease, HAC, ocular disease, phenobarbital administration, PCV, WBC, basal serum T4, post-TSH serum T4, and serum TSH concentrations were included in subsequent forward conditional multiple logistic regression analyses. As depicted in Table 5, only basal serum T4 concentration ( $B = -11.64$ ,  $OR < 0.0001$ , 95% CI =  $<0.0001-0.0290$ ,  $P = 0.0049$ ) and the presence of concurrent HAC ( $B = -3.493$ ,  $OR = 0.0304$ , 95% CI =  $0.0013-0.7242$ ,  $P = 0.0308$ ) were associated with the diagnosis of hypothyroidism, indicating a dog having low basal serum T4 concentration has a high possibility of hypothyroidism and a dog with the concurrent HAC has low possibility of hypothyroidism.

The ROCs of the basal serum T4 concentration in all the dogs included in the present study were analyzed.

The ROC AUC was 0.5900 (95% CI = 0.4165–0.7579), but optimal cut-off of basal serum T4 concentration between hypothyroid and non-hypothyroid dogs could not be determined due to non-statistical significance of the ROC AUC ( $P = 0.3313$ ). Because the presence of HAC could negatively affect the diagnosis of hypothyroidism considering the results of previous multiple logistic regression analyses, the dogs having concurrent HAC were excluded. After exclusion of HAC dogs, the ROC AUC was 0.8299 (95% CI = 0.7022–0.9576,  $P = 0.0004$ ) (Figure 3). The optimal cut-off of basal serum T4 concentration between hypothyroid and non-hypothyroid dogs was determined graphically to be 0.8640  $\mu\text{g/dL}$ , with the sensitivity of 94.12% (95% CI = 71.31–99.85%) and the specificity of 60.87% (95% CI = 38.54–80.29%). At a cut-off point of  $< 0.4500 \mu\text{g/dL}$ , sensitivity and specificity were 47.06% (95% CI = 22.98–72.19%) and 86.96% (95% CI = 66.41–97.22%), respectively. At a cut-off point of  $< 0.9950 \mu\text{g/dL}$ , sensitivity and specificity were 100% (95% CI = 80.49–100%) and 43.48 % (95% CI = 23.19–65.51%), respectively.

**Table 1** Signalment and concurrent disease of evaluated dogs in the hypothyroid, intermediate, and euthyroid groups.

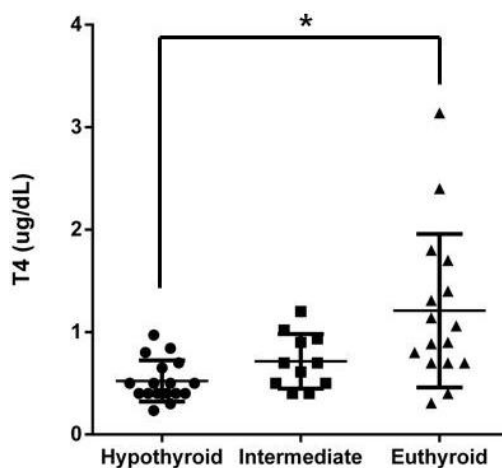
	Hypothyroid (n=17)	Intermediate (n=11)	Euthyroid (n=16)	P value
Age	13 (10-15)	13 (1-17)	7 (1-16)	0.0034 <sup>#</sup>
Body weight	5.53 (2.36-27.5)	4.3 (1.4-8.92)	4.78 (2.3-10.95)	0.3239
Sex				0.8527
M	3 (17.6%)	0 (0%)	3 (18.8%)	
CM	6 (35.3%)	4 (36.4%)	5 (31.3%)	
F	3 (17.6%)	2 (18.2%)	3 (18.8%)	
SF	5 (29.4%)	5 (45.5%)	5 (31.3%)	
BCS	6 (2-8)	5 (3-8)	5 (3-7)	0.4608
Concurrent diseases*				
CVHD	7 (41.2%)	6 (54.5%)	4 (25%)	0.2900
Neurology	3 (17.6%)	2 (18.2%)	10 (62.5%)	0.0109 <sup>#</sup>
Dermatology	5 (29.4%)	2 (18.2%)	3 (18.8%)	0.7026
GI tract	2 (11.8%)	1 (9.1%)	3 (18.8%)	0.7413
Anemia	4 (23.5%)	3 (27.3%)	1 (6.3%)	0.2910
Diabetes mellitus	2 (11.8%)	1 (9.1%)	0 (0%)	0.3839
HAC	3 (17.6%)	4 (36.4%)	0 (0%)	0.0387 <sup>#</sup>
CKD	5 (29.4%)	4 (36.4%)	4 (25%)	0.8168
Tumor	2 (11.8%)	1 (9.1%)	0 (0%)	0.3839
Muscular	0 (0%)	0 (0%)	1 (6.3%)	0.4085
Ocular	3 (17.6%)	0 (0%)	0 (0%)	0.0776 <sup>#</sup>
Medications				
Glucocorticoid	1 (5.9%)	0 (0%)	2 (12.5%)	0.4401
Phenobarbital	0 (0%)	1 (9.1%)	6 (37.5%)	0.0102 <sup>#</sup>

M, male; CM, castrated male; F, female; SF, sprayed female; BCS, body condition score; CVHD, chronic valvular heart disease; GI tract, gastrointestinal tract; HAC, hyperadrenocorticism; CKD, chronic kidney disease.

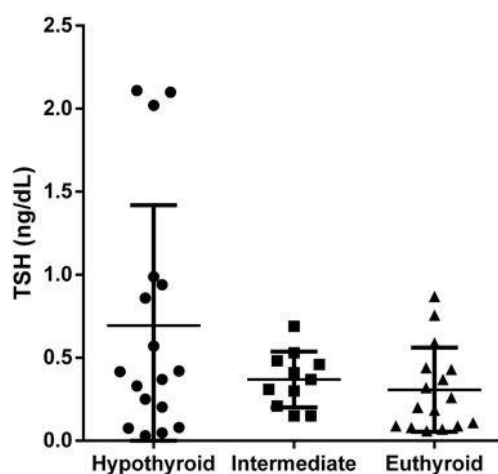
Results of the age, body weight, and BCS are expressed as median (range).

\*The number (percent) of each concurrent disease are the repeated frequency of the dogs.

<sup>#</sup>Variables with  $P < 0.20$  are included in the subsequent multiple logistic regression analyses (Table 5).



**Figure 1** Scatterplot of basal serum T4 concentrations in the hypothyroid (n=17), intermediate (n=11), and euthyroid (n=16) groups. Dogs were classified based on the results of the TSH stimulation test.  
\*Differences between the hypothyroid and euthyroid groups were statistically significant ( $P = 0.0012$ ).



**Figure 2** Scatterplot of basal serum TSH concentrations in the hypothyroid (n=17), intermediate (n=11), and euthyroid (n=16) groups. Dogs were classified based on the results of the TSH stimulation test.  
There were no statistically significant differences among the three groups.

**Table 2** Case distribution according to the basal serum T4 concentration of three groups.

Basal serum T4 concentration	Hypothyroid	Intermediate	Euthyroid
<0.5 $\mu\text{g/dL}$ (n=12)	8 (66.6%)	2 (16.6%)	2 (16.6%)
0.5 to 1.0 $\mu\text{g/dL}$ (n=22)	9 (40.9%)	7 (31.8%)	6 (27.3%)
1.0 to 1.5 $\mu\text{g/dL}$ (n=6)	0 (0%)	2 (33.3%)	4 (66.6%)
1.5 to 2.0 $\mu\text{g/dL}$ (n=2)	0 (0%)	0 (0%)	2 (100%)
>2.0 $\mu\text{g/dL}$ (n=2)	0 (0%)	0 (0%)	2 (100%)

T4, thyroxine.

**Table 3** Comparison the results between the basal T4 combined with the basal TSH concentrations in three groups.

	Hypothyroid	Intermediate	Euthyroid
Low T4, High TSH (n = 13)	7 (53.8%)	3 (23.1%)	3 (23.1%)
Low T4, Normal TSH (n = 21)	10 (47.6%)	6 (28.6%)	5 (23.8%)
Normal T4, High TSH (n = 3)	-	1 (33.3%)	2 (66.6%)
Normal T4, Normal TSH (n = 7)	-	1 (14.3%)	6 (85.7%)

T4, thyroxine; TSH, thyroid stimulating hormone.

Reference interval of T4 and TSH concentration were 1 to 4  $\mu\text{g/dL}$  and 0.05 to 0.42 ng/mL, respectively.

The range of low T4 were < 1  $\mu\text{g/dL}$  and the range of high TSH were > 0.42 ng/mL.

**Table 4** Comparison the laboratory findings in dogs included in this study.

	Hypothyroid group (n = 17)				Intermediate group (n = 11)				Euthyroid group (n = 16)				P value
	N	Value	Below RI	Above RI	N	Value	Below RI	Above RI	N	Value	Below RI	Above RI	
RBC [5.65-8.87*10 <sup>6</sup> /μL]	15	5.52 (4.72-6.43)	9 (60%)	0 (0%)	9	5.95 (4.72-6.37)	4 (44.4%)	0 (0%)	16	6.26 (5.95-7.01)	3 (18.8%)	0 (0%)	0.3199
PCV [37.3-61.7%]	16	35.18 (26.72-43.64)	9 (56.3%)	0 (0%)	9	36.72 (26.60-46.84)	4 (44.4%)	0 (0%)	16	40.82 (32.68-48.96)	3 (18.8%)	0 (0%)	0.1871 <sup>#</sup>
WBC [5.05-16.76*10 <sup>3</sup> μL]	15	10.93 (9.70-15.27)	0 (0%)	3 (20%)	9	8.35 (6.99-11.67)	0 (0%)	1 (11.1%)	16	9.74 (7.04-14.99)	0 (0%)	3 (18.8%)	0.1944 <sup>#</sup>
Platelet [148-484*10 <sup>3</sup> /μL]	15	468.3 (284.9-651.7)	1 (6.7%)	9 (60%)	9	527.8 (317.2-738.4)	0 (0%)	4 (44.4%)	16	550.8 (266.6-835.0)	0 (0%)	7 (43.8%)	0.6132
Cholesterol [135-270mg/dL]	9	301.0 (187.5-463.0)	0 (0%)	5 (55.6%)	8	296.0 (138.3-360.3)	1 (12.5%)	5 (62.5%)	8	255.5 (243.8-288.5)	0 (0%)	3 (37.5%)	0.7866
Triglyceride [21-116mg/dL]	8	73.5 (50.5-100.8)	0 (0%)	1 (12.5%)	5	77.0 (49.0-100.5)	0 (0%)	0 (0%)	4	86.5 (78.75-106.3)	0 (0%)	0 (0%)	0.6720
ALT [21-102IU/L]	14	60.5 (38.75-103.3)	1 (7.1%)	3 (21.4%)	10	106.5 (52.0-156.0)	0 (0%)	6 (60%)	12	82.0 (32.0-146.3)	0 (0%)	4 (33.3%)	0.4007
ALP [29-97IU/L]	15	442.0 (211.0-1040)	0 (0%)	13 (86.7%)	10	759.0 (202.3-2613)	0 (0%)	10 (100%)	14	370.0 (186.8-1443)	0 (0%)	14 (100%)	0.8830
BUN [7-25mg/dL]	16	30.25 (18.75-44.23)	1 (6.3%)	10 (62.5%)	11	26.6 (19.5-39.0)	1 (9.1%)	7 (63.6%)	14	17.05 (9.83-45.63)	1 (7.1%)	5 (35.7%)	0.4394
Creatinine [0.5-1.5mg/dL]	16	1.10 (0.70-1.48)	0 (0%)	3 (18.8%)	11	1.10 (0.70-1.40)	0 (0%)	2 (18.2%)	14	0.80 (0.48-1.33)	3 (21.4%)	2 (14.3%)	0.3408
Glucose [65-118mg/dL]	6	100.0 (86.5-374.0)	0 (0%)	2 (33.3%)	6	112.5 (91.75-306.0)	0 (0%)	2 (33.3%)	6	119.0 (80.5-191.3)	1 (16.7%)	3 (50%)	0.9464
Total protein [5.4-7.1g/dL]	14	6.70 (5.95-6.90)	1 (7.1%)	2 (14.3%)	11	6.30 (6.10-6.40)	1 (9.1%)	0 (0%)	14	6.30 (6.08-7.03)	2 (14.3%)	2 (14.3%)	0.5527
Albumin [2.6-3.3g/dL]	14	2.95 (2.47-3.43)	3 (21.4%)	3 (21.4%)	11	3.06 (2.61-3.52)	1 (9.1%)	1 (9.1%)	14	3.04 (2.49-3.59)	2 (14.3%)	5 (35.7%)	0.8346

Results are expressed as mean (± SD) or median (interquartile range), respectively, for normally and non-normally distributed variables.

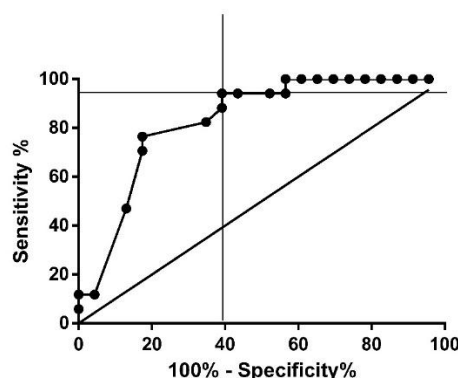
RBC, red blood cell; PCV, packed cell volume; WBC, white blood cell; ALT, alanine aminotransferase; ALP, alkaline phosphatase; BUN, blood urea nitrogen.

<sup>#</sup> Variables with  $P < 0.20$  are included in the subsequent multiple logistic regression analyses (Table 5)

**Table 5** Results of multiple logistic regression analyses of basal serum T4 concentration with confounding variables in diagnosis of hypothyroidism in dogs.

Variables	B	OR	95% CI	P value
Basal serum T4	-11.64	<0.0001	<0.0001-0.0290	0.0049
HAC	-3.493	0.0304	0.0013-0.7242	0.0308

CI, Confidence interval; HAC, hyperadrenocorticism; T4, thyroxine; OR, odds ratio.

**Figure 3** Receiver operating characteristic curve illustrating the sensitivity and specificity for the use of basal serum T4 concentration to distinguish hypothyroid dogs from non-hypothyroid dogs. The thick diagonal line represents a completely uninformative test, wherein the area under the curve is 50%. The area under the curve of the receiver operating characteristic curve represented by the thicker line is 0.8299 (95% CI = 0.7022-0.9576). The point of intersection indicates optimal cut-off of 0.8640  $\mu\text{g/dL}$  for the differentiation between hypothyroid and non-hypothyroid dogs, with corresponding sensitivity and specificity of 94.12% (95% CI = 71.31-99.85%) and 60.87% (95% CI = 38.54-80.29%), respectively.

## Discussion

In the present study, the utility of basal serum T4 and TSH combination test in diagnosing canine hypothyroidism was evaluated by comparing it with TSH stimulation test. The use of any other medication and concurrent diseases were not considered reasons for exclusion because the present study was aimed to examine the usefulness of basal T4/TSH in diagnosing hypothyroidism in a general canine population under a clinical situation, and the present study confirmed that hypothyroidism based on TSH stimulation test results can distinguish hypothyroidism from NTIS (Daminet, *et al.* 2006; Scott-Moncrieff, *et al.*, 2015). Twenty-five percent of all cases (11 of 44 cases) and 28.9% of cases with pre-administration T4 concentration below 1.0  $\mu\text{g/dL}$  (9 of 34 cases) were classified into the intermediate group. To distinguish the intermediate group from the other two groups, laboratory findings among the three groups were compared, but there were no significant differences.

This study reported a significant difference in the basal T4 concentration between the hypothyroid and euthyroid groups. Because low basal T4 concentrations were found in all three groups, it was difficult to diagnose hypothyroidism based on basal T4 concentration alone. Of the 16 cases diagnosed as euthyroid, 8 cases had serum T4 concentration lower than 1.0  $\mu\text{g/dL}$ . Among them, 6 cases had been administered phenobarbital, 1 case had severe CKD (International Renal Interest Society stage 4), and 1 case had concurrent diseases (CKD, chronic valvular heart disease, and canine atopic dermatitis). Nine of 11 cases in the intermediate group had basal T4 concentrations lower than 1  $\mu\text{g/dL}$ , and they had been administered phenobarbital or had concurrent diseases (CKD or

HAC). Changes in thyroid hormone levels caused by certain medications or by concurrent diseases have been reported and include the following pathophysiology: decreased TSH secretion, decreased synthesis of T4, decreased binding affinity of proteins, inhibition of the de-iodination of T4 to T3, or any combination of these (Scott-Moncrieff, 2015; Weisinga and Van den Berghe, 2013). The abovementioned can lead to low basal T4 concentrations, and these dogs could be misdiagnosed with hypothyroidism so results should be carefully interpreted. Nevertheless, the lower the basal T4 concentration, the more likely it is to be diagnosed as hypothyroidism. Of the 34 cases with a basal T4 concentration of less than 1.0  $\mu\text{g/dL}$ , 50% of cases were diagnosed as hypothyroidism and 23.5% of cases as euthyroid. But of the 20 cases with a basal T4 concentration of less than 0.7  $\mu\text{g/dL}$ , 65% of cases were diagnosed as hypothyroidism and 10% of cases as euthyroid. On the other hand, basal T4 concentrations were also useful to reject hypothyroidism as a diagnosis. In the present study, the maximum basal T4 concentrations in the hypothyroid and intermediate groups were 0.97 and 1.20  $\mu\text{g/dL}$ , respectively. There were no dogs diagnosed with hypothyroidism when basal T4 was higher than 1.0  $\mu\text{g/dL}$ , and all dogs were diagnosed as euthyroid when basal T4 level was higher than 1.2  $\mu\text{g/dL}$ .

A previous study had reported that serum TSH levels were lower than 0.6 ng/mL in 39 euthyroid dogs and that this could be a useful tool for diagnosing hypothyroidism (Boretti and Reusch, 2004). In this study, the serum TSH levels reached 0.87 ng/mL in the euthyroid group, and there were no significant differences among the three groups, which is inconsistent with the previous study. However, the serum TSH concentrations in the intermediate and

euthyroid groups were relatively low compared to that of the hypothyroid group, and the 75<sup>th</sup> percentiles of the intermediate and euthyroid groups were close to the upper limit of the reference range of serum TSH level. Moreover, only 3 of 27 cases in the intermediate and euthyroid groups had TSH concentrations higher than 0.6 ng/mL. Of these 3 cases, 2 cases were normal basal T4 levels, and only 1 case was found to be low basal T4 concentration (0.9 µg/dL). In addition, among the cases with TSH level higher than 0.6 ng/mL, all cases diagnosed as hypothyroidism were identified as basal T4 levels lower than 0.7 µg/dL. Therefore, it would be inappropriate to diagnose hypothyroidism based on serum TSH levels alone, but it can be helpful in the interpretation of the basal serum T4 and TSH combination test.

According to the literature, the combination of reduced T4 and elevated TSH levels provides a sensitivity of 63-67% and a specificity of 98-100% in diagnosing canine hypothyroidism (Ferguson, 2007; McCann, 2015). However, because the present study was conducted to examine the diagnostic usefulness of basal T4 and TSH combination test for the diagnosis of hypothyroidism in dogs with and without concurrent disease(s) in clinical practice, the accuracy of the T4 and TSH combination test in diagnosing hypothyroidism was lower than that reported in previous studies. This study only included cases that had undergone the TSH stimulation test, and the test was performed when it was difficult to evaluate the thyroid function with the basal T4 and TSH combination test because the patient was suspected of NTIS. Therefore, our cases had concurrent diseases or had been administered drugs that could affect the thyroid hormone metabolism. Dogs diagnosed as intermediate and euthyroid had chronic valvular heart disease, CKD, neurological disease, HAC, anemia, or a combination of those. Moreover, in the present study, the classification into euthyroid, hypothyroid, or intermediate was based on TSH stimulation test results. Thus, sensitivity and specificity of the basal T4 and TSH combination test could not be assessed, and comparison with previous studies may be inappropriate. In the present study, the diagnostic test accuracy of the basal T4 and TSH combination test was 2.45, indicating the test's potential usefulness. Nevertheless, the results of this study suggest that there is a high probability (22.2%) of false positives associated with the basal T4 and TSH combination test in patients suspected of NTIS. Canine hypothyroidism mainly has a middle-age onset, so it is likely for dogs to have concurrent diseases or to be medicated with drugs that influence thyroid function. Therefore, in the dog suspected with NTIS, it may be effective to consider administering thyroid drugs if a combination of T4 level less than 0.7 µg/dL and TSH level greater than 0.6 ng/mL in the basal T4 and TSH combination test, and if not, proceed the TSH stimulation test.

Multiple logistic regression analyses in the present study revealed that the presence of HAC could influence the diagnosis of hypothyroidism. Three of 17 hypothyroid dogs and 4 of 27 non-hypothyroid dogs had concurrent HAC. Of the 3 hypothyroid dogs with concurrent HAC, two dogs had normal TSH concentrations. However, of the 4 non-hypothyroid

dogs with concurrent HAC, two dogs had elevated TSH although the dogs were classified into intermediate group based on the results of TSH stimulation test. Therefore, in hypothyroid dogs with concurrent HAC, diagnosis of hypothyroidism could be established on the basis of TSH stimulation test, and not on the basis of basal T4 and TSH combination test. In practice, it can be difficult to differentiate hypothyroidism from HAC in dogs due to some clinical similarities. HAC dogs frequently have low basal serum T4 concentrations due to the possible mechanisms, including inhibition of TSH secretion and reduced serum protein binding of T4 as observed in the present study (Kemppainen *et al.*, 1983; Peterson *et al.*, 1984; Kenefick and Neiger, 2008). Furthermore, 62% of HAC dogs with low T4 concentration had low free T4 concentration, indicating that serum free T4 also has low diagnostic accuracy in hypothyroidism in HAC dogs (Ferguson and Peterson, 1992). Fortunately, a previous study revealed that TSH concentrations were increased following trilostane treatment in HAC dogs (Kenefick and Neiger, 2008), suggesting hypothyroidism could be more accurately diagnosed or ruled out following HAC stabilization. In addition, all hypothyroid dogs with concurrent HAC revealed post-TSH T4 concentrations below 1.4 µg/dL, but those concentrations of non-hypothyroid dogs with concurrent HAC were above 1.9 µg/dL which can be considered normal thyroid function (Corsini *et al.*, 2021). Thus, TSH stimulation test can differentiate hypothyroidism from non-hypothyroidism in HAC dogs. Above all, when HAC dogs are suspected with concurrent hypothyroidism, TSH stimulation test can be considered. Alternatively, basal thyroid function might be re-tested after HAC stabilization. Further longitudinal study is necessary to clarify the alteration of thyroid hormone concentrations in HAC dogs following treatment.

On the basis of our results, algorithm using basal T4/TSH concentration for diagnosis of hypothyroidism might be suggested. Basal T4 concentration is a screening test useful for its high sensitivity (Peterson *et al.*, 1997), indicating hypothyroidism can be ruled out when a dog has normal T4 concentration. Furthermore, basal T4 cut-off of 0.8640 µg/dL presented sensitivity of 94.12% and specificity of 60.87% after HAC dogs were excluded in the present study. All non-hypothyroid dogs without HAC had basal T4 concentration > 0.8640 µg/dL, thus hypothyroidism can be also ruled out when a non-HAC dog has basal T4 concentration above the cut-off. Conversely, two non-hypothyroid dogs had basal T4 concentration < 0.8640 µg/dL; the dogs had basal T4 concentrations of 0.7 and 0.4 µg/dL and TSH concentration of 0.43 and 0.44 ng/mL, respectively. Thus, the dogs could be misdiagnosed with hypothyroidism if only basal T4/TSH concentration was used. Fortunately, all the dogs were euthyroid based on the results of TSH stimulation test. One of the dogs had received phenobarbital to manage idiopathic epilepsy. In the univariate analysis, phenobarbital could be considered to affect the diagnosis of hypothyroidism although the drug may not affect the diagnosis of hypothyroidism after subsequent multivariate analysis. Phenobarbital has been



considered as a drug that decreases serum T4 and increases TSH concentration (Daminet *et al.*, 2003). Serum T4 can decrease to levels observed in hypothyroid dogs, and TSH concentrations can elevate slightly although the TSH concentration remains  $< 0.7$  ng/mL in all dogs administered phenobarbital over long term (Müller *et al.*, 2000), which was also observed in our case. However, 24% of hypothyroid dogs have a basal TSH concentration within the reference range (Peterson *et al.*, 1997). Consequently, basal T4 and TSH combination test may not useful to diagnose or exclude hypothyroidism in a dog receiving phenobarbital. TSH stimulation test should be performed in a dog receiving phenobarbital and exhibiting low basal T4 ( $< 0.8640$  µg/dL) with elevated TSH concentration. Alternatively, basal T4/TSH concentration should be re-evaluated at least 4–6 weeks after cessation of phenobarbital administration (Daminet *et al.*, 2003).

Another dog with low basal T4 ( $< 0.8640$  µg/dL) and elevated TSH concentration had anemic CKD. Furthermore, 2 of 3 anemic CKD dogs had low basal T4 with normal TSH concentrations, indicating non-hypothyroidism. Consequently, basal T4 and TSH combination test cannot be used to diagnose or exclude hypothyroidism in anemic CKD dogs. Hypothyroidism in dogs can reduce their renal function via several effects in dogs (Scott-Moncrief, 2015; Di Paola *et al.*, 2021). In addition, non-regenerative anemia is frequently observed as a clinical manifestation in older dogs with hypothyroidism or CKD. In clinical practice, several scenarios can be possible; low thyroid level as NTIS secondary to anemia and/or CKD; or concurrent hypothyroidism and CKD with or without hypothyroidism affecting renal functions. Therefore, if possible, TSH stimulation test should be performed without delay in anemic CKD dogs with low basal T4 ( $< 0.8640$  µg/dL) and elevated TSH concentration. Alternately, thyroid supplementation is cautiously considered in such dogs because the time taken to manage anemic CKD and normalize thyroid function in dogs is undetermined.

After the exclusion of HAC, phenobarbital administration and anemic CKD, the sensitivity and specificity of a low basal T4 ( $< 0.8640$  µg/dL) with a high TSH were 70% (95% CI = 45.72–88.11%) and 100% (95% CI = 73.54–100.0%), respectively. Consequently, the diagnosis of hypothyroidism using basal T4 and TSH combination test in dogs with HAC and dogs administered phenobarbital should be postponed until HAC stabilization and at least 4–6 weeks after the discontinuation of phenobarbital, respectively. In anemic CKD dogs, it can be recommended that TSH stimulation test should be performed without delay, or thyroid supplementation with close response monitoring should be considered after careful interpretation of the history, clinical signs, clinicopathologic results and thyroid function test results. Therefore, if a dog has low basal T4 ( $< 0.8640$  µg/dL) with a high TSH after exclusion of HAC, anemic CKD and phenobarbital administration, it should receive thyroid supplementation cautiously. The abovementioned algorithm may be applied to dogs in clinical practice, but a further large cohort study needs to be conducted to adjust or revise the suggested algorithm.

Univariate analysis revealed that neurologic diseases could affect the diagnosis of hypothyroidism when using basal T4/TSH concentration, but consequent multivariate analysis concluded that the neurologic diseases may not affect the diagnosis of hypothyroidism. The reason for this unexpected result is not known, but there is a possible explanation. In some instances, neurologic deficits may be the sole manifestation of hypothyroidism. Consequently, the diagnosis of the neurologic disorders associated with hypothyroidism can be challenging (Bertran *et al.*, 2013). Therefore, some dogs showing neurologic signs could be candidates for the thyroid function tests. However, 10 of 15 dogs with neurologic diseases were euthyroid, and the dogs were completely differentiated after TSH stimulation test in the present study. In addition, the dogs with neurologic diseases in the intermediate and euthyroid group can be completely ruled out following the abovementioned algorithm by using basal T4/TSH concentrations.

Hypothyroidism is not an all-or-nothing disease. Generally, it is caused by lymphocytic thyroiditis or idiopathic atrophy and is characterized by gradual loss of thyroid function (Lucke *et al.*, 1983). This leads to unclear and ambiguous results sometimes. The TSH stimulation test is recognized as the gold standard for the diagnosis of canine hypothyroidism (Daminet *et al.*, 2007), but it is difficult to evaluate thyroid function in the intermediate group through this test. Laboratory test results were compared to distinguish the intermediate group from the other two groups, but the results of the present study did not identify any significant differences among the three groups. In dogs classified into the intermediate group, the clinical trial of thyroid drugs or the regular follow-up of the thyroid function may be realistic options. However, since dogs in the intermediate group may progress to hypothyroidism, further studies on the intermediate group are needed to evaluate the thyroid function more accurately.

There were several limitations to the present study. As the total cases were divided into three groups, the number of cases in each group was relatively small. Due to the possibility of errors caused by a small sample, the interpretation of the results should be cautious. Second, this study only compared the results between the basal serum T4 and TSH combination test and the TSH stimulation test and did not classify the cases in the intermediate group as hypothyroid or euthyroid. Follow-up studies analyzing histopathologic results of thyroid biopsies or complete recovery without thyroid hormone supplementation might be needed. If the cases in the intermediate group were confirmed to be euthyroid or hypothyroid, a more intuitive evaluation based on the basal T4 and TSH combination test could be suggested.

In conclusion, in this study, no dogs with normal T4 concentration were diagnosed as hypothyroid, while low basal T4 concentrations were found in euthyroid dogs. TSH concentrations above the reference range were found in all three groups. The basal T4 and TSH combination test was useful to reject canine hypothyroidism, but it was not useful to confirm true hypothyroidism. Therefore, in dogs with concurrent systemic diseases or receiving drugs

known to affect the thyroid hormone metabolism, the TSH stimulation test is recommended to confirm hypothyroidism.

### Acknowledgements

The authors would like to thank all the owners of the dogs included in this study. This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No. NRF-2021R1F1A1061799).

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