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Qualitative and semiquantitative assessment of thyroid hormone binding proteins in greyhounds and other dog breeds

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ABSTRACT

Total thyroxine (T4) concentrations are lower in healthy greyhounds compared to most other non-sighthound breeds. In humans, variations in the structure or concentration of the major thyroid hormone binding proteins are responsible for most reported differences between total T4 concentrations in healthy individuals from different ethnic groups or other subpopulations. The aim of this study was to determine if such variations are also responsible for the lower total T4 concentrations in greyhounds. The predicted protein sequences of thyroxine-binding globulin (TBG), transthyretin and albumin were determined in liver tissue from a euthyroid greyhound with decreased T4 concentration and a Jack Russell terrier using reverse-transcriptase PCR. Sequences were compared to each other and online reference sequences. Serum proteins from 21 greyhounds and 21 non-sighthound dogs were separated by denaturing electrophoresis and immunoblots probed with polyclonal antibodies to human TBG and transthyretin. Reactive bands were quantified by densitrometry, expressed relative to the mean of reference samples included in each gel. Serum albumin concentrations were measured using a commercially-available assay. Several SNPs were identified but none was thought likely to explain the lower total T4 concentrations in greyhounds. There was no significant difference between the quantity of any of the binding proteins in serum from greyhounds and non-sighthound dogs. However, total T4 and transthyretin concentrations were highly correlated in the greyhound group ($r = 0.73$, $P = 0.0002$). Variation in the sequence of thyroid hormone binding proteins is not responsible for low greyhound total T4 concentrations. Further evaluation of the role of transthyretin is warranted.

1. Introduction

Interbreed variation in thyroid hormone concentrations is well-recognized in dogs [1–3]. In particular, healthy greyhounds, considered as sighthounds, display markedly decreased total thyroxine (T4) concentrations in comparison to most other non-sighthound breeds. Values are below non-breed-specific reference intervals in more than

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90%, and below the limit of detection of most assays in 30%, of dogs [3]. Free T4 values are also significantly lower, but maintained within reference interval in approximately 80% of greyhounds [3]. The cause of this interbreed variation is unknown, but it has clinical importance because it can lead to an erroneous diagnosis of hypothyroidism in greyhounds [4].

In humans, over 99 % of circulating T4 is bound to specific proteins: T4-binding globulin (TBG); transthyretin; and albumin [5]. Of these, TBG binds the largest proportion (49%–68%) of T4, with highest affinity. Transthyretin and albumin bind approximately 11%–13% and 7%–20% of T4, respectively, both with lower affinities. Functions of these binding proteins include limitation of urinary hormone loss, buffering against sudden fluctuations in thyroid gland secretion, provision of an extracellular, rapidly available reservoir of thyroid hormone and possibly facilitation of thyroid hormone transport across cell membranes [5,6]. Variations in the structure or concentration of these binding proteins are responsible for most of the reported differences in total T4 concentrations between different ethnic groups and other subpopulations [6]. However, because of the predominance of T4-binding to TBG, only abnormalities of this protein result in markedly decreased total T4 concentrations. Over 30 variants of TBG have been described at the molecular level; the majority linked to mutations in the gene encoding TBG (*SERPINA7*; Gene ID 6906). These variants are not typically associated with altered thyroid function. Although an altered quantity of protein-bound hormone clearly affects total T4 concentration, metabolically active free T4 and thyroid stimulating hormone (TSH) concentrations are typically within reference interval, and affected patients remain euthyroid.

In dogs, over 99 % of T4 is also protein bound and it has been estimated that TBG, transthyretin and albumin bind 12%–60%, 12%–39% and 16%–40.4% of T4, respectively [7–9]. The wide range in reported results between studies likely reflects differences in methodology. The overall T4-binding affinity of these proteins (principally due to TBG) is thought to be lower in dogs compared to humans, likely explaining the lower total T4 concentration and higher free T4 fraction observed in this species [10]. Canine TBG has been described as a 75 kDa protein that lacks immunological cross-reactivity with its human orthologue [10]. Canine transthyretin circulates in plasma as a tetramer of non-covalently bound monomers and displays immunological cross-reactivity with the human protein [11–13].

It has been hypothesized that variations in the sequence or concentration of thyroid hormone binding proteins could also be at least partly responsible for the observed differences in total T4 concentration between greyhounds and other non-sighthound breeds [2–4]. This is supported by the proportionally larger difference in total compared to free T4 concentrations observed in this breed. Because of the relatively large proportion of T4 binding to transthyretin and albumin in dogs in some studies, it is possible that, by contrast to humans, variants of any of the three major proteins could contribute to decreased total T4 concentrations within this breed. The aim of the current study was to determine if differences in the sequence or quantity of the major thyroid hormone binding proteins was responsible for the decreased total T4 concentration observed in greyhounds compared to most other non-sighthound breeds.

2. Materials and methods

2.1. Determination of thyroid hormone binding protein transcript coding sequences

Sequences of TBG, transthyretin and albumin were determined using liver tissues collected post-euthanasia from a 6-year-old greyhound. Although healthy based on clinical and routine clinicopathological assessment, the greyhound was euthanased at the owner's request because of the development of meningoencephalitis in multiple offspring. Thyroid histopathology was unremarkable in this dog. Sequences were also determined using liver obtained from a Jack Russell terrier euthanased for reasons unrelated to the study. In both dogs, tissue was collected within 10 minutes of euthanasia and snap frozen in liquid nitrogen and stored at -80C. Total RNA was isolated and cDNA prepared as previously described [14].

The predicted sequences of canine TBG (XM_538128.2), transthyretin (XM_537290.2) and albumin (XM_850464.1) transcripts were retrieved from the NCBI database (National Center for Biotechnology Information (NCBI) Available at: https://www.ncbi.nlm.nih.gov. Accessed: February 10, 2010). However, because of poor quality or incomplete 5'UTR, the sequence of the 5' UTR reported for the corresponding human transcripts (M14091, NM_00371.3 and NM_000477, respectively) were also retrieved from the NCBI database and compared to an identical length of upstream sequence of canine gDNA retrieved from the Ensembl database (Ensembl genome browser, release No. 54. Available at: www.ensembl.org/index.html. Accessed February 10, 2010) (ENSCAFT00000028383, ENSCAFT00000035370 and ENSCAFT00000004843, respectively). The entire transcript sequences were predicted by combining the information obtained from these sources. Primers were designed using the online software Primer3 (http://frodo.wi.mit.edu/primer3) and are detailed in Supplementary Table 1.

All PCRs were performed in 40µL reaction volumes with 28.0µl H_2O, 5.6µl BDM, 2µl 20µM forward primer, 2µl 20µM reverse primer, 0.4µl Platinum Taq Polymerase (Platinum Taq DNA Polymerase, Invitrogen) and 2µl of template. Most PCR reactions consisted of a 5-minute denaturation stage at 95C, followed by 35 cycles of denaturation (95 °C for 30s), annealing (60 °C for 30s) and extension (72 °C for 60s). A final extension stage (72 °C for 10 minutes) was applied at the end of each reaction. The annealing temperature of the ALBORF_F1R1 primer pair was increased to 62 °C to optimize the specificity of the reaction. The remainder of the PCR reaction was as described above. After PCR, amplicons were purified, followed by Sanger sequencing using an ABI 3730xl DNA Analyzer (Thermo Fisher Scientific) at GATC Biotech. Coding sequences were determined, and nucleotides numbered sequentially from the start codon. The cDNA sequences from the two dogs were aligned and compared to each other and the reference boxer sequence using the CLUSTALW general-purpose

multisequence alignment program (https://www.ebi.ac.uk/Tools/msa/clustalw2).

2.2. Quantitative/semiquantitative assessment of thyroid hormone binding proteins

Blood samples for protein quantification were collected from 21 healthy greyhounds. Ten dogs were male and 11 female. Ages ranged from six months to 12 years (mean 32.5 months). Thyroid hormone concentrations were assessed as previously described [3]. Values below the limit of detection of the assay were assigned a value equal to the limit of detection. Remaining aliquots of serum were stored at -20 °C.

Serum samples from 21 healthy, non-sighthound dogs were also used. These samples were submitted to the Michigan State University Veterinary Diagnostic Laboratory as part of a screening program for hypothyroidism, using assays validated for use in the dog [15–18]. Samples were included from a variety of breeds: three each of great Dane and mastiff, two each of Labrador retriever, golden retriever, Rhodesian ridgeback, standard poodle, rottweiler, and one each of Shetland sheepdog, Tibetan terrier, coton de tulear, giant schnauzer and Dobermann. Six dogs were male and 15 were female. Ages ranged from 1.5 to 5 years (mean age 30.9 months). Samples from Michigan State University were shipped overnight on ice to University College Dublin, and frozen at -20 °C on arrival.

The cDNA sequences for canine TBG were translated to protein sequences using the ExPASy translate tool (www.expasy.ch/tools/dna.html). The signal peptide cleavage site was predicted using SignalP-5.0 Server (http://www.cbs.dtu.dk/services/SignalP) to be between the 19th and 20th amino acid sequences (probability 1.0), which would result in a secreted 395 amino acid protein, identical in length to its human orthologue. The predicted canine TBG protein sequence was next aligned to the human sequence (NP_000345.2) using CLUSTALW. A pairwise alignment score of 82 was calculated. Next, the human secreted TBG protein sequence was compared against all non-redundant canine protein sequences using BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The top result returned was canine TBG, with an 83% identity reported between the human and canine proteins. The next highest scoring protein was α1-antiproteinase, with a reported identity of 53%. Potential antigenic sites within the human and canine TBG protein sequences were also predicted using the Mobyle@pasteur online tool (http://mobyle.pasteur.fr/cgi-bin/portal.py). Several identical predicted antigenic sequences were present in both the human and canine proteins, including three of the four highest scoring predicted human antigenic sequences (KNALALFVLPK, TIMVLVNYIHFKA and RSILFLGKVVNP). Based upon these findings, it was postulated that the canine and human proteins would display antigenic cross reactivity, and that polyclonal anti-human TBG antibodies would detect the canine protein.

To test this hypothesis, protein immunoblots were probed with a primary polyclonal anti-human TBG antibody and secondary HRP conjugated antibody. A single band at approximately 51 kDa was observed in both canine and human serum samples (Supplementary Fig. 1), which was believed to represent canine TBG. To assess the ability of a protein in this region to bind T4, a protein immunoblot was performed using anti-T4 primary antibodies. The results demonstrated T4 binding to a protein of approximately 70 kDa (presumably albumin), a protein with an apparent molecular weight of approximately 51 kDa, and several proteins of lower molecular weight (Supplementary Fig. 2).

Serum concentrations of TBG and transthyretin were determined using a semiquantitative Western blot approach. Serum proteins were denatured by heating serum samples to 100C in a water bath for ten minutes in the presence of an equal volume of 2X denaturing loading buffer (100mM Tris HCl pH 6.8; 2% β-mercaptoethanol; 4% SDS; 0.2% bromophenol blue; 20% glycerol). Samples were diluted by adding 5µL of denatured sample to 65µL of 1X denaturing loading buffer. A larger aliquot was prepared from a pool of serum from 10 individual dogs of various breeds. This standard sample was included in duplicate on each gel.

One-dimensional SDS PAGE was performed using a discontinuous buffer system. Samples for semi-quantitative assessment of TBG and transthyretin were separated by 12 and 15 % acrylamide gel electrophoresis, respectively, at fixed voltage (160V) for approximately 70 minutes in a tris-glycine running buffer (250mM Tris base, 192 mM glycine, 1% SDS) or until the loading buffer had reached the bottom of the gel. Lanes were prepared as follows: 2.5µL of SeeBlue Pre-Stained Standard (Thermo Fisher Scientific) was placed in lane 1; 15µL of denatured, diluted pooled serum (reference sample) was placed in lane 2 and in the last lane; 15 µL of denatured, diluted greyhound or non-sighthound sera were placed in the central lanes. Greyhound and non-sighthound samples were alternated in each gel to minimize bias from uneven transfer.

For immunodetection, proteins were transferred to a polyvinylidine fluoride (PVDF) membrane using an electrophoretic transfer cell and a Tris glycine based buffer containing methanol (25mM Tris base; 192mM glycine; 20% methanol) with a fixed 100V over 90 minutes. Following transfer, the PVDF membrane was placed in a blocking solution containing 5 % dry skimmed milk powder in phosphate PBS containing 0.1% Tween 20 for one hour. The membrane was washed briefly in PBS Tween, and then incubated with primary and secondary antibody (Supplementary Table 2). The membrane was washed three times over 15 minutes following both the primary and secondary antibody incubation steps.

After the final wash, membranes were incubated for five minutes with SuperSignal West Pico Chemiluminescent substrate (Pierce). Immunoblots were imaged using Visionworks LS image acquisition and analysis software (UVP). For semi-quantitative analysis of serum thyroid hormone binding proteins, a region of interest (ROI) was drawn around each of the protein bands and an identically sized area of background (Supplementary Fig. 3). Signal intensity per unit area was calculated for each ROI, and the background value subtracted from the total value for each band. The coefficient of variation (CV) between the two identical reference samples on each gel was then

Table 1
Thyroid hormone concentrations in the greyhound and non-sighthound dogs

	Greyhound for coding sequence determination	Greyhound group (n = 21)	Non-sighthound group (n = 21)
Total T4 (nmol/L)	6.4	6.4 (6.4–23.9)	31.5 (9.91)
Free T4 (pmol/L)	6.0	4.9 (3.2–36.1)	16.1 (5.07)
Total T3 (nmol/L)	0.7	1.1 (0.34)	1.4 (0.8–3.2)
cTSH (ng/mL)	0.1	0.12 (0.04–0.86)	0.17 (0.102)

Mean (standard deviation) and median (range) and are reported for parametric and non-parametric data, respectively.

Table 2
Variations in the greyhound and Jack Russell terrier coding sequences in comparison to the online reference sequence

Transcript	Position	SNP	Amino acid substitution	Breed
TBG	526	A/G	Met - Val	Jack Russell
Transthyretin	61	A/G	Ser – Gly	Greyhound
	315	C/T	Synonymous	Greyhound
Albumin	27	C/T	Synonymous	Greyhound and Jack Russell
	1075	G/T	Ala - Ser	Greyhound and Jack Russell
	1422	A/T	Glu - Asp	Greyhound and Jack Russell

Table 3
Thyroxine binding globulin (TBG) and transthyretin relative optical densities and albumin concentrations in 21 greyhound and 21 non-sighthound dogs

	Greyhound		Non-sighthound		*P* value
	Median	Range	Median	Range	
TBG	0.98	0.57–1.79	1.06	0.45–1.87	0.4395
Transthyretin	0.74	0.15–1.20	0.80	0.49–1.59	0.2202
Albumin (g/L)	32.4	23.7–37.6	35.0	28.4–40.7	0.0388
	Mean	SD	Mean	SD	
Log TBG	1.00	0.023	1.00	0.033	0.8546
Log transthyretin	0.97	0.052	0.99	0.031	0.0501

Log transformed TBG and transthyretin values are also included. Both TBG and transthyretin values represent the relative optical density on Western blot expressed as a ratio to the mean of the two standards included within each gel. There was no significant difference between greyhound and non-sighthound values for any parameter.

calculated and results were included only if the CV was less than 15%. Protein immunoblots with higher CV values were repeated. Background corrected signal intensity values were expressed as a proportion of the mean of the two standards to allow comparison of values from different immunoblots.

The coefficients of variation for TBG and transthyretin background-corrected intensities were 12.8 and 12.6 %, respectively, determined from 10 and 9 standards included on single gels, respectively. The observed/expected values at 90, 80, 70, 60, 50, 40, 30 and 20 % of the standard concentration were 102.4, 77.9, 79.7, 78.9, 77.3, 86.8, 90.8 and 102 %, respectively for TBG. When log transformation ($f(x)=\log_{10}(x)$) was applied to observed and expected counts, these percentages measured 100.2, 97.9, 98.1, 98.0, 97.8, 98.8, 99.1 and 100.2 %, respectively. Observed / expected ratios for transthyretin were 109.0, 105.4 120.0, 121.3, 131.4, 127.1, 130.7 and 124.4 %, respectively. Log transformed values were 100.9, 100.5, 101.9, 102.1, 103.0, 102.7, 103.1 and 102.6 %, respectively.

Serum albumin concentrations were measured using a bromocresol green-based method within the Randox RX Imola system (Randox Laboratories).

This study was performed in accordance with the contemporaneous guidelines of the UCD Animal Research Ethics Committee.

2.3. Statistical analysis

Data were tested for normality using the D'Agostino and Pearson method. Parametric and non-parametric data were reported as mean (standard deviation) and median (range), respectively. Values between groups were compared using the unpaired t-test (log transformed TBG and transthyretin values) and Mann Whitney test (albumin, TBG and transthyretin values) for parametric and non-parametric data, respectively. For comparison of TBG, transthyretin and albumin values between the two groups, a *P* value of 0.01 (Bonferroni adjusted *P* value = 0.05/5) was considered significant. Correlation between total T4 concentration and quantity of each of the three binding proteins was evaluated using the Spearman rank correlation test. All statistical analyses were performed using commercially available software (GraphPad Prism (version 5.01, San Diego, CA).

3. Results

In the greyhound used for determination of thyroid hormone binding protein coding transcript sequences, total and free T4 (equilibrium dialysis) concentrations were below, and total T3 and cTSH concentrations within, non-breed-specific reference intervals (6.4 (15–50) nmol/L;

6.0 (6.6–40) pmol/L; 0.7 (0.7–2.5) nmol/L; 0.1 (0–0.68) ng/mL, respectively. In the greyhounds used for thyroid hormone binding protein quantification, total and free T4 concentrations were below the lower limit of the reference interval in 18 and 11 of the 21 greyhound samples, respectively (Table 1). These results were considered typical of the breed. By contrast, all thyroid hormone results from the non-sighthound dogs were within their respective reference intervals.

The greyhound TBG coding sequence was identical to the boxer sequence (Table 2). The Jack Russell terrier TBG sequence contained an A/G non-synonymous single nucleotide polymorphism (SNP) at position 526. This results in conservative substitution of methionine for valine at this site. The greyhound transthyretin sequence contained two SNPs; an A/G non-synonymous SNP at position 61 (serine/glycine; a semi-conservative substitution) and a synonymous C/T SNP at position 315. The SNP at position 61 has been previously identified in the poodle (Ensembl genome browser, release No. 54). The Jack Russell terrier transthyretin sequence was identical to the boxer sequence. The greyhound and Jack Russell terrier albumin coding sequences were identical but differed from the boxer sequence at three locations. A C residue was identified at position 27 in comparison to a T residue in the reference boxer sequence; a C/T synonymous SNP has been previously identified at this location in the boxer. A G/T non-synonymous SNP was identified in the greyhound and Jack Russell terrier at position 1075 (alanine/serine). An A/T non-synonymous SNP was also identified in both dogs at position 1422 (glutamic acid/aspartic acid). Both of these amino acid substitutions are conservative.

Relative optical densities of canine TBG and transthyretin were compared in the greyhound and non-sighthound groups using a semiquantitative immunoblot approach. There was no significant difference between the two groups before or after transformation of optical density data (Table 3 and Fig. 1). There was also no significant difference in albumin concentrations between the two groups. Total T4 concentrations were significantly correlated with the relative optical densities of transthyretin in the greyhound ($P = 0.0002$, $r_s = 0.73$) but not the non-sighthound ($P = 0.23$) group (Fig. 2). There was no significant correlation between total T4 and albumin concentrations in either group ($P = 0.45$ and $P = 0.47$, respectively), or between total T4 concentrations and TBG relative optical densities ($P = 0.12$ and $P = 0.28$, respectively).

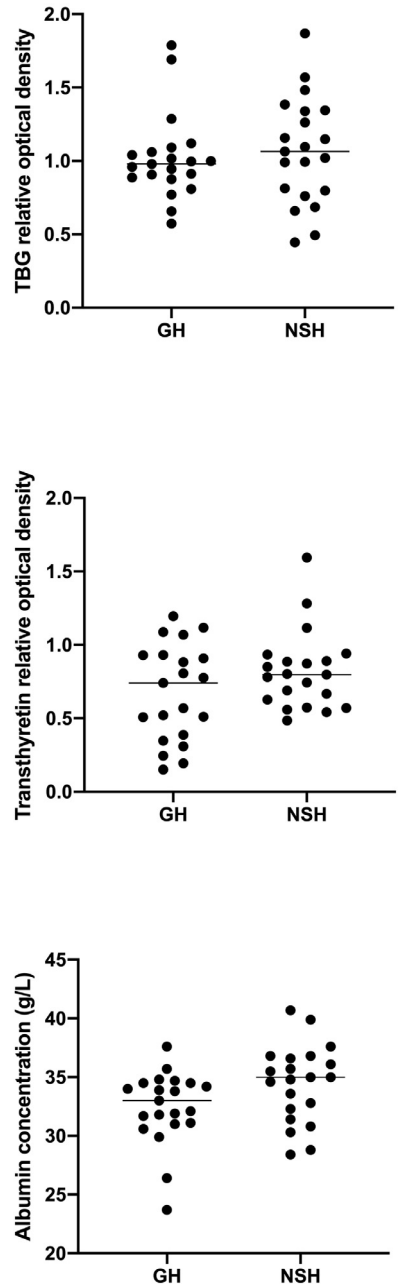

Fig. 1. Thyroxine binding globulin (TBG) and transthyretin relative optical densities and albumin concentrations in 21 greyhound (GH) and 21 non-sighthound (NSH) dogs (A, B and C, respectively). TBG and TTR values represent the relative optical density on Western blot expressed as a ratio to the mean of the two standards included within each gel. There was no significant difference between greyhound and non-sighthound concentrations for any of the three thyroid hormone binding proteins. The horizontal line represents the median value in each group.

4. Discussion

The current study identified several previously unreported SNPs in canine TBG, transthyretin and albumin cDNA sequences. Several SNPs were non-synonymous and, by definition, would result in alterations of the amino acid sequence. Although it is possible that some of the identified SNPs are associated with altered T4 binding, their presence in both greyhound and non-sighthound breeds makes it unlikely that they are primarily responsible for the observed differences in T4 concentration between greyhounds and most other non-sighthound breeds.

All of the non-synonymous SNPs identified in the current study were associated with either conservative or semi-conservative amino acid substitutions. Conservative amino acid substitutions are characterized by the replacement of one amino acid residue by another that possesses similar chemical properties, for example charge or hy-

drophobicity [19]. In a semi-conservative amino acid substitution, the amino acid is replaced by another that has similar steric conformation but does not share chemical properties. Conservative and semi-conservative substitutions are less likely to result in significant alterations of protein function when compared to non-conservative substitutions. However, this is not always true as exemplified by the decreased serum T4 binding in human patients expressing TBG-Quebec; a TBG variant characterized by a C/T non-synonymous SNP that leads to a conservative amino acid substitution (replacement of histidine 331 by tyrosine) [20]. Therefore, further functional protein studies are required to determine the potential clinical significance of canine thyroid hormone binding protein variants in different breeds.

In the current study, cDNA sequences from only one greyhound were determined. This dog had thyroid hormone values considered typical for the breed (decreased total and free T4 and reference interval T3 and cTSH concentrations). Due to the high level of intrabreed genetic homogeneity, it is unlikely that analysis of a larger number of greyhound cDNA sequences would have identified a potentially causative variation [21].

A limitation of the current study is that only cDNA sequences were assessed. It is possible that post-translational modifications could alter the function of thyroid hormone binding proteins. In humans, TBG undergoes extensive post-translational glycosylation. Although these residues are not necessary for the function of the protein *per se*, they are required for tertiary folding, secretion and stability of the TBG molecule [22]. Additional proteomic studies would be necessary to investigate potential post-translational variants. In addition, although the complete open reading frames open of TBG, transthyretin and albumin were reported in the current study, the entire 5'UTRs and 3'UTRs were not sequenced. Variations in UTR would not lead to altered protein sequence, but could lead to altered gene expression. It would be possible to determine the complete coding sequence by performing 5' and 3' rapid amplification of cDNA ends (RACE). It is also possible that other genetic factors, such as variation in the TSH receptor, deiodinase or related genes, are responsible for the difference in T4 concentrations between greyhounds and most other non-sighthound breeds [23].

There was no significant difference between any of the measured thyroid hormone binding protein concentrations in greyhounds and non-sighthound groups. However, total T4 concentrations were strongly correlated with transthyretin relative optical density in the greyhound group. All 11 of the greyhounds with total T4 concentrations below the limit of detection of the assay had transthyretin values below the median of this group. By contrast, transthyretin values were above the median in all three greyhounds with reference interval total T4 concentrations. Therefore, it is possible that lower transthyretin concentrations contribute to the lower total T4 concentrations in some greyhound dogs. However, the magnitude of the difference between total T4 concentrations in greyhound and non-sighthound breeds could not be explained by reduced concentrations of transthyretin alone, unless this protein binds a much higher proportion of T4 than previously estimated. As thyroid hormone binding affinity is affected by numerous factors including pH, temperature and a variety of chemicals, in vitro models of proportional hormone binding are difficult to interpret [7,24,25].. Additional studies are indicated to assess the relative importance of the major serum binding proteins in dogs.

It was postulated that the band identified at 51 kDa in canine serum represented canine TBG. By contrast, a previous study estimated the apparent molecular weight of canine TBG to be 75 kDa, based upon the position of the purified protein following 1D SDS PAGE, and described a lack of immunological cross reactivity between the human and canine proteins [10]. The assumption of the current study was based upon the high level of identity between the human and predicted canine protein sequences, the presence of several shared predicted antigenic determinants, the similar position of the human and protein bands following 1D SDS PAGE and the identification of a band in similar position with capability of binding T4 and the absence of protein with apparent molecular weight of 75kDa with T4-binding capability.

In humans, most studies report the apparent molecular weight of human serum TBG to be 54 kDa [26,27]. The separation of proteins using SDS PAGE is based upon the movement of proteins relative to their charge to mass ratio. Sodium dodecyl sulfate is an anionic detergent that binds to polypeptides, conferring a negative charge and destroying most of the complex structures within the protein. Peptides bind SDS in proportion to their relative molecular mass. During SDS PAGE, the relative migration distance of a protein (R_f) is almost solely dependent on its molecular weight (R_f is negatively proportional to the log of its mass). If proteins of known mass (standard markers) are run simultaneously with the unknown proteins, the relationship between R_f and mass can be plotted, and the masses of unknown proteins estimated. However, the migration of glycoproteins such as TBG is far less predictable. The presence of carbohydrate side chains (that do not bind SDS) increases the molecular weight and decreases the charge/mass ratio. For this reason, the apparent molecular weight of human TBG in various SDS PAGE studies has varied from 54 to over 60 kDa, presumably depending in part upon the extent of original glycosylation and degree of deglycosylation during sample preparation [27–29]. The percentage of acrylamide used in the experiment can also influence migration [30]. Therefore, the difference in apparent molecular mass noted in the current study compared to previous reports was thought likely to reflect methodological differences.

Polyclonal anti-human TBG antibodies were used to detect canine TBG in the current study. It has been previously reported that human and canine TBG lack immunological cross reactivity [10]. In that study, crossed immunoelectrophoresis of both canine serum and purified TBG was performed in agarose containing antisera to the human analogue of this protein. No precipitation lines were apparent when either purified canine TBG or canine serum was used, suggesting an absence of immunochemical identity. By contrast, in the current study, a band in the corresponding position to the human protein was consistently identified. Regardless of the identity of this band, such

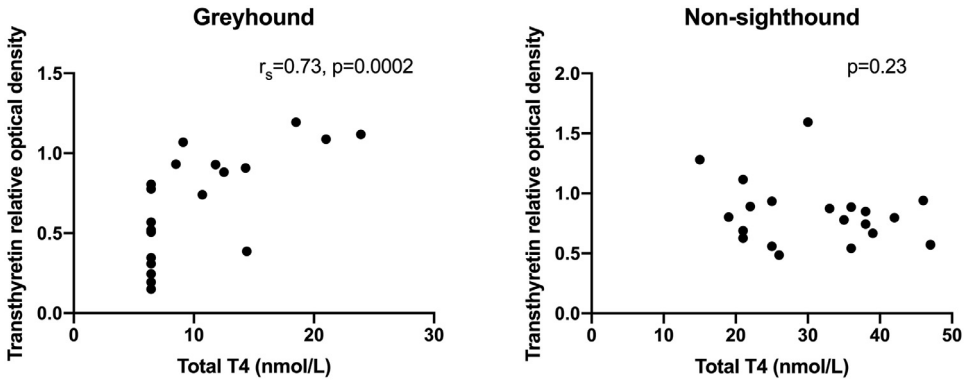

Fig. 2. Correlation between total thyroxine (T4) concentrations and transthyretin relative optical densities in greyhound and non-sighthound serum.

an immunological reaction should have been detected by crossed immunoelectrophoresis of canine serum with anti-human TBG antibodies. The reason for this discrepancy is unclear. Although both studies utilized polyclonal antisera, these can vary in efficacy, depending upon the methods of immunization of the host animal and methods of antibody harvesting and purification. No information was provided regarding the type of antiserum used in the previous study.

There are several limitations to the use of semi-quantitative immunoblots as a method of protein concentration estimation. Although the procedure was standardized, differences between individually cast gels and in the efficacy of protein transfer and antibody applications invariably leads to difficulty in comparing results from different immunoblots. Quantification methods can also introduce numerous errors due to variability in image acquisition, selection of bands and the method of computer-aided determination of the optical density [31]. Despite these limitations, the inclusion of standards as a method of semi-quantification of protein on immunoblots has been described in numerous other reports [32–35]. In the current study, the inclusion of two identical reference samples within each gel was used as a method of standardizing concentrations and was also used to evaluate for uneven transfer or staining during the procedure. In addition, greyhound and non-sighthound samples were alternated on every gel. Despite these precautions, semi-quantitative immunoblot analysis is inherently less precise and accurate when compared to immunoassay techniques. Given the immunological cross reactivity between the human and canine major thyroid hormone binding proteins, it may be possible to validate the use of human assays for the measurement of canine TBG and transthyretin. This would greatly facilitate the study of thyroid hormone binding protein variations and abnormalities within this species, and help to further explore the association between transthyretin and total T4 concentrations in greyhounds and other breeds.

CRediT authorship contribution statement

R.E. Shiel: Conceptualization, Methodology, Writing - original draft. **C.M. Nolan:** Formal analysis, Writing - review & editing. **J.E. Nally:** Methodology, Formal analysis. **K.R. Refsal:** Methodology, Conceptualization. **C.T. Mooney:** Supervision, Writing - review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.domaniend.2021.106623.

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