CANINE THYROID FUNCTION:
A CLINICAL AND CLINICOPATHOLOGICAL INVESTIGATION

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For the degree of DOCTOR OF PHILOSOPHY

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December 1999

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ABSTRACT

This study evaluated commercially available tests of thyroid function in dogs. The diagnostic utility of these tests for hypothyroidism are reported, as are the epidemiological, clinical, biochemical and haematological features of the disease. The clinical and clinicopathological changes associated with treatment for hypothyroidism are reported and a strategy for therapeutic monitoring is provided. The value of thyroid function tests as prognostic markers in non-thyroidal illness (NTI) is evaluated.

Commercial kit assays for total 3,5,3',5'- tetraiodothyronine (thyroxine, T_4), free T_4, total 3,5,3'-triiodothyronine (T_3), endogenous canine thyrotropin (thyroid stimulating hormone, TSH) (cTSH) and thyroglobulin autoantibodies (TgAb) were successfully validated for use with canine serum and subsequently evaluated in 140 dogs with clinical signs suggestive of hypothyroidism. Hypothyroidism was confirmed (n=52) or excluded (n=88) based on the results of bovine TSH response tests. Median circulating total T_4 (4.7 nmol/L), free T_4 (3.22 pmol/L) and total T_3 (1.32 nmol/L) concentrations were significantly decreased and cTSH (1.38 ng/ml) significantly increased in hypothyroid dogs compared to the corresponding values of 20.1 nmol/L, 13.84 pmol/L, 2.38 nmol/L and 0.27 ng/ml, respectively in euthyroid dogs with NTI. Optimal diagnostic cut-off values for total T_4, free T_4, total T_3 and cTSH for the confirmation of hypothyroidism were 14.8 nmol/L, 6.66 pmol/L, 1.77 nmol/L and 0.69 ng/ml, respectively. The corresponding test sensitivities and specificities for hypothyroidism were 0.96 and 0.70 (total T_4), 0.81 and 0.93 (free T_4), 0.76 and 0.80 (total T_3), and 0.79 and 0.82 (cTSH), respectively. Transient increases in cTSH values in euthyroid dogs were usually due to recovery from NTI or the recent use of sulphonamide-containing drug therapy. Concurrent illness may reduce cTSH concentrations into the euthyroid range in hypothyroid dogs.

No age, gender, neutering status or breed predilections for hypothyroidism were identified. Although hypothyroidism occurred in dogs of any age, it was rare in animals less than two years old. Evaluation of breed and pedigree status was hindered by inherent biases within the population studied. However, the most commonly affected breed-types were retrievers, spaniels, terriers, crossbreeds and doberman pinschers although these were also common amongst the dogs with NTI. No effect of pedigree status on the likelihood of developing hypothyroidism was identified. The most common clinical features associated with hypothyroidism were metabolic signs (85 % cases), particularly lethargy (77 %), weight gain (42 %) or exercise intolerance (27 %), and dermatological signs (79 %), particularly hair thinning (56 %), poor coat quality (29 %), hyperpigmentation (19 %) and superficial pyoderma (15 %). Concurrent metabolic and dermatological signs occurred in 67 % cases. The most common routine biochemical and haematological abnormalities compared with the laboratory reference range were increases in circulating triglyceride (88 % cases), cholesterol (78 %) and fructosamine (43 %) concentrations and reduced red blood cell count (40 %). However similar abnormalities frequently occurred in euthyroid dogs and the routine tests which most efficiently confirmed hypothyroidism were reduced red blood cell count and increased fructosamine values.

Successful clinical resolution of hypothyroidism was achieved by once daily administration of synthetic L-thyroxine therapy. Metabolic signs usually improved within two weeks after starting therapy whereas dermatological improvements took longer. Mean body weight decreased by 10 % in treated cases during the first three months of therapy. Most dogs were essentially clinically normal within 13 weeks of starting therapy. Six hour post-pill total T_4 concentrations were a reliable method of objectively assessing therapeutic control. Corresponding mean values in well controlled cases were 55 nmol/L whereas values less than 35 nmol/L were usually associated with inadequate clinical improvement. Circulating cTSH estimation was of limited utility for therapeutic monitoring although

increased concentrations usually predicted the need for an increase in therapy. Improvements in numerous biochemical and haematological parameters occurred during therapy and were consistent with metabolic normalisation. However, only creatinine estimation could be used to differentiate between dogs which were adequately or inadequately clinically controlled. Iatrogenic thyrotoxicosis occasionally occurred but was uncommon and easily treated.

Circulating TgAb was negative, equivocal or positive in 19 (45 %), eight (19 %) and 15 (36 %) of 42 hypothyroid, 55 (79 %), 11 (16 %) and four (6 %) of 70 healthy, and 72 (94 %), five (6 %) and none, of 77 euthyroid dogs with NTI, respectively. TgAb positive and equivocal results were significantly more common in hypothyroid than euthyroid dogs. Two of four apparently healthy TgAb-positive dogs had additional biochemical abnormalities consistent with profound primary hypothyroidism. The high prevalence of false-positive TgAb results obtained in previous reports was not a significant problem with the method evaluated, and a TgAb positive result was highly suggestive of thyroid disease.

Clinical features, and thyroid hormone parameters were determined in 205 dogs with varying types and severity of illnesses. Mean (± standard deviation (s.d.)) age (years) was significantly lower in dogs which made a full recovery (5.6 ± 4.3) compared to those which were made a partial recovery (7.4 ± 3.7 years) or which were euthanatised (7.6 ± 3.6 years). Dogs with acute diseases were significantly less likely to survive than those with chronic illnesses. Dogs with neoplasia were significantly less likely to survive than other dogs, whereas orthopaedic cases were significantly more likely to make a full recovery. The most common pattern of thyroid hormone abnormalities was reduced total T_3 alone (approximately 40 % cases) or concurrently reduced total T_4 and total T_3 (approximately 30 % cases) concentrations. Reduction in total T_4 values alone was rare. Circulating total and free T_4 concentrations were significantly decreased by certain drug therapies but these did not influence prognosis. Circulating total T_4, free T_4 and total T_3 were significantly decreased in dogs that died compared to those which survived. The magnitude of depression was inversely correlated to the likelihood of survival. The maintenance of normal circulating total T_3 concentrations was a strong predictor that an animal would survive.

This clinical, clinicopathological and epidemiological study has greatly increased the knowledge and understanding of canine thyroid function in hypothyroidism and NTI. The findings have improved the ability to diagnose, treat and prognosticate, and have opened the door for future research.

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ACKNOWLEDGEMENTS

Much of the work performed in this thesis was funded by a British Small Animal Veterinary Association (BSAVA) Petsavers award. Without this funding there is no doubt that the study could not have been performed. I would like to acknowledge the financial support provided by the BSAVA and hope that the proven commitment of this association to veterinary clinical research has been appropriately rewarded by the advancement in knowledge outlined herein. Numerous individuals have given me support in one way or another throughout the period of study and preparation of this manuscript. Firstly, I would like to thank Peter Graham, whose exceptional enthusiasm for clinical research, specifically clinical biochemistry and endocrinology initially sparked my own interest. His attention to detail and ability to apply his knowledge to a wide range of disciplines is one which I will always admire and never match. His astute recognition of the important from the unimportant in all areas of life is a talent which he has in enviable abundance. I hope that we spend as many happy hours in the future pondering the relative merits of tests of canine thyroid function at the same time as whether to go home or go to Clatties, as we have in the past. Brian Wright, as the senior Departmental administrator has unfailingly used his magic to provide me with all the tools that I have required to perform the study. Alongside the unequaled enthusiasm of Professor Max Murray and the generous support that he has afforded me through the Department of Veterinary Clinical Studies, Brian's ability to supply airline tickets and pay hotel bills has made my position at Glasgow one that students at other institutions would only envy. I hope that the presentation of the results throughout the world, has enhanced the reputation of Glasgow University and the Veterinary School specifically. The boys in the biochemistry lab, particularly James Harvie, David Moffat and Chris McComb, simultaneously provided invaluable technical guidance and spectacular humour. I will always remember them. The nursing staff in the school all assisted me with cases, but particular thanks go to Pam who always made herself available, was unfailingly supportive and invariably was left to sort out my mistakes. Stuart Reid had provided invaluable statistical guidance and been a true buddy in and out of the water. David Irvine has also been generous with his time and advice and helped me out of many of the statistical holes that I have frequently dug. The routine biochemistry and endocrinology technicians have diligently and professionally performed all the routine biochemistry and haematology analyses detailed in the study. Additionally, Chris McComb performed nearly all the endocrine analyses detailed in Chapter 8 at exceptionally short notice. His

willingness to still be at work after 3 am was invaluable in completing the work on time and I am deeply grateful to him. I would like to thank Ian Jeffcoate for providing a number of the samples used in Chapter 3, and Betty Aughey, Lizzie Gault and Jan MacDonald for assisting in the collection of the Bearded Collie samples used in Chapter 7. Diagnostic Products Corporation generously supplied the cTSH assays used in Chapters 3 and 4. I would also like acknowledge the assistance of Oxford Laboratories, Nichols Institute Diagnostics, Diagnostic Products Corporation and Chiron Diagnostics for freely supplying the assays used in Chapter 8. Without their generous support that invaluable section of this thesis would not have been possible. The clinical case data collected in Chapter 8 required considerable assistance from the clinical staff in the Department of Veterinary Clinical Studies. Without exception they gave their time generously and I thank them for their patience. It is clear that without the cooperation of the owners of the dogs studied in this thesis, the work would have been impossible. I thank them all for their patience and their patients.

My principal supervisor and friend, Carmel Mooney has guided, directed, suggested and demanded all in variable amounts, but always as required. Her depth of knowledge in the subject area is unparalleled and to receive such committed supervision must be truly exceptional. I will always be grateful. Alongside Peter, it was Carmel who encouraged me to start the study, and provided the support and backing within and outside the University to ensure that I was funded. Without doubt her continued encouragement and knowledge has been responsible for quantity and quality of the novel data which has resulted from this study, and the timely preparation of the thesis. I hope she is justly proud of the final result.

Jan and Roggie have spent many evenings alone in the name of my PhD. The time has come to start going home at nights and I am looking forward to every minute of my repayment.

Richard Dixon, December 1999

AUTHOR'S DECLARATION

The work presented in this thesis was performed solely by the author except where the assistance of others has been acknowledged.

Richard M Dixon, December 1999

PUBLICATIONS AND PRESENTATIONS

Some of the work in this thesis has been the subject of the following publications and presentations:

Publications

Dixon R. M., Reid S.W.J. & Mooney, C. T. (1999) Epidemiological, clinical and routine clinicopathological features of canine hypothyroidism. *The Veterinary Record* **145**, 481-487.

Dixon R. M. & Mooney, C. T. (1999) Canine serum thyroglobulin autoantibodies in health, hypothyroidism and non-thyroidal illness. *Research in Veterinary Science* **66**, 243-246.

Dixon R. M. & Mooney, C. T. (1999) An evaluation of serum free thyroxine and thyrotropin concentrations in the diagnosis of canine hypothyroidism. *Journal of Small Animal Practice* **40**, 72-78.

Dixon R. M., Graham P.A. & Mooney, C.T. (1996) Serum thyrotropin concentrations: A new diagnostic test for canine hypothyroidism. *The Veterinary Record* **138**, 594-595.

Dixon, R. M., McComb, C. & Mooney, C. T. (*In Preparation*) The relationship between thyroid function and clinical outcome in a referral population of dogs with nonthyroidal illness.

Dixon, R. M. & Mooney, C. T. (*In Preparation*) Determination of clinical prognostic markers in a canine referral population.

Dixon, R. M. & Mooney, C. T. (*In Preparation*) Therapeutic monitoring and routine biochemical and haematological findings in treated hypothyroid dogs.

Dixon, R. M., McComb, C. & Mooney, C. T. (*In Preparation)* Circulating canine thyroglobulin autoantibodies in nonthyroidal illness.

Presentations

Dixon R. M. & Mooney, C. T. (1999) Thyroglobulin autoantibodies: another piece in the canine hypothyroidism jigsaw puzzle. *Proceedings of the British Small Animal Veterinary Association (BSAVA) annual congress p254*

Dixon, R. M. (1999) Endocrinology in Practice. Annual VetCPD Conference, 8th-9th June 1999, Stirling.

Dixon, R. M. & Mooney, C. T. (1999) New diagnostic methods in the evaluation of canine thyroid disease. Proceedings of the 4th European Comparative Clinical Pathology Meeting, 1-4th June 1999, Verona, Italy, p47-48.

Dixon, R. M. (1999) Clinical Endocrinology. BSAVA/BVNA 14th Annual Scientific Weekend, 7th-9th May 1999, Crieff

Dixon, R. M. (1999) Clinical endocrinology. BVNA annual congress, Stonleigh, Warwickshire, Oct 1999.

Dixon R.M., Reid, S.W.J. & Mooney, C. T. (1998) Serum fructosamine concentrations in hypothyroid dogs. *Proceedings of the British Small Animal Veterinary Association (BSAVA) annual congress p257*

Dixon R. M. & Mooney, C. T.(1998). Validation of a free thyroxine assay for use with canine serum and its application in the diagnosis of hypothyroidism. *Proceedings of the Association of Veterinary Teachers and Research Workers (AVT&RW) annual scientific meeting p62*

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Dixon R. M., Reid, S.W.J., & Mooney, C. T.(1998) Serum fructosamine concentrations in hypothyroid dogs *Proceedings of the World Small Animal Veterinary Association (WSAVA) annual congress p257*

Dixon R. M., & Mooney, C. T. (1998) Use of a novel thyroglobulin autoantibody immunoassay for detection of thyroid disease in the dog. Annual meeting of the animal clinical chemistry association/association for comparative haematology. Macclesfield, November 1998

Dixon R. M. & Mooney, C. T. (1997), Interaction between glucocorticoids and endogenous thyrotropin in the dog. *Proceedings of the Association of Veterinary Teachers and Research Workers (AVT&RW) annual scientific meeting p13*

Dixon R. M. Nachreiner, R.F., Refsal, K. R., Graham, P.A. & Mooney, C. T.(1997) 24 hour diurnal variation in canine thyrotropin (TSH) concentrations in euthyroid, untreated hypothyroid, and treated hypothyroid dogs. *Proceedings of the British Small Animal Veterinary Association (BSAVA) annual congress p243.*

Dixon R. M. & Mooney, C. T. (1997) (poster), Effect of exogenous glucocorticoids on serum thyrotropin concentrations in the dog. *European Comparative Clinical Pathology Association 3rd biannual congress, Breda, Netherlands*

Dixon, R. M. (1997) Hypothyroidism in Practice. Evening BVA regional meeting, 1997, Ayrshire.

Dixon R. M., P. A. Graham, Harvie, J. & Mooney, C. T.(1997) Comparison of endogenous serum thyrotropin (cTSH) concentrations with bovine TSH response test results in euthyroid and hypothyroid dogs. *Proceedings of the 15th American College of Veterinary Internal Medicine (ACVIM) forum p668, Lake Buena Vista, FL*

Dixon R. M., Mooney, C. T., Graham, P. A. & Harvie J.(1996) Preliminary results of a novel canine thyrotropin immunoradiometric assay. *Animal Clinical Chemistry Association (ACCA) spring meeting (29-30 March 1996), St Ives*

Dixon R. M., Graham P. A., Mooney,C. T. (1996). Canine serum TSH measurement in the diagnosis of hypothyroidism. *Proceedings of the Association of Veterinary Teachers and Research Workers (AVT&RW) annual scientific meeting p50.*

Dixon R. M., Graham P.A., Mooney C.T., (1996). Canine serum TSH measurement in the diagnosis of hypothyroidism. *Proceedings of the British Small Animal Veterinary Association (BSAVA) annual congress p219.*

Dixon R. M., Graham, P. A. Harvie, J. & Mooney, C. T. (1996) Comparison of canine thyrotropin (TSH) concentrations with bovine TSH response test results in 38 dogs. *Proceedings of the 7th International Society for Animal Clinical Biochemistry (ISACB) annual congress p9, Glasgow*

DEDICATION

To climbing on an ice covered Scottish mountain with Luke Arnott

since there are fewer greater privileges or people

CHAPTER 1
INTRODUCTION

1.1 BACKGROUND

Hypothyroidism is the clinical syndrome caused by reduced circulating concentrations of the active thyroid hormones T_3 and T_4. It is widely acknowledged as one of the most common endocrine diseases affecting dogs. In dogs, hypothyroidism usually results from primary thyroid gland failure, caused by either lymphocytic thyroiditis or idiopathic atrophy (Kemppainen & Clark, 1994). Central hypothyroidism, whereby inadequate thyrotropin (thyroid stimulating hormone, TSH) secretion causes thyroidal insufficiency also occasionally occurs in dogs.

In humans, the diagnosis of primary hypothyroidism can be accurately and reliably achieved by measurement of circulating free (non protein-bound) T_4 and human TSH concentrations (Stockigt, 1996). By contrast, achieving a reliable diagnosis of canine hypothyroidism is fraught with difficulty. Measurement of circulating total T_4 concentration is often recommended but is complicated by the overlap of results in hypothyroidism, in health and in NTI (Belshaw & Rinjberk, 1979; Nelson, Ihle, Feldman & Bottoms, 1991; Peterson, Melian & Nichols, 1997; Scott-Moncrieff, Nelson, Bruner & Williams, 1998). Free T_4 is considered to be the principal determinant of cellular hormone activity and consequently should provide a more accurate indicator of true thyroid status (Robbins & Rall, 1957). However, estimation of free T_4 can be performed by a variety of techniques and the most widely used of these methods in veterinary medicine have been severely criticised on both theoretical and practical grounds (Ekins, 1985). Measurement of free T_4 by tracer equilibrium dialysis is considered to be the gold standard method but this technique is cumbersome and unsuitable for most veterinary laboratories.

Recently assays for cTSH, free T_4 measured by a modified equilibrium dialysis kit technique, and TgAb have become commercially available. At the commencement of this study, there were no reports regarding cTSH measurement in the investigation of spontaneous hypothyroidism; very limited data had been published on reliably measured free T_4 concentrations in suspected hypothyroid dogs; and no reports of the clinical utility of TgAb estimation for hypothyroidism using the recently commercialised assay were available. In particular, very little is known about the effect of non-thyroidal factors such as NTI or commonly used drug therapies on these tests. During the course of this study, Jensen, Iverson, Høier, Kristensen & Henriksen, (1996) reported relatively poor diagnostic

sensitivity of the cTSH assay for hypothyroidism but thyroid function itself was not reliably studied. Peterson *et al.,* (1997) documented hypothyroidism in 54 dogs, and reported improved diagnostic accuracy of free T_4 by modified equilibrium dialysis compared to total T_4 measurement. Peterson *et al.,* (1997) also evaluated the cTSH assay but reported relatively poor sensitivity for hypothyroidism. Scott-Moncrieff *et al.,* (1998) evaluated the same assay as Peterson *et al.,* (1997) with broadly similar conclusions, but cases with borderline thyroid function based on TSH response tests were excluded from that study. Nachreiner, Refsal, Graham, Hauptman & Watson, (1998) evaluated a kit for TgAb estimation but the study was laboratory based and only limited clinical information was available from the cases.

Epidemiological information on canine hypothyroidism worldwide is sparse, and as it applies to the UK specifically, essentially non-existent. Previous studies of the disease have been limited by variable or poor quality methods for reliably confirming hypothyroidism, and studies performed elsewhere cannot necessarily be presumed to reflect the pattern of hypothyroidism in the population of dogs in the United Kingdom (UK) at this time.

Another consequence of the difficulties encountered in reliably confirming hypothyroidism, is that published data of the clinical and clinicopathological features of the disease are often sparse and must be interpreted cautiously. Hypercholesterolaemia and a mild anaemia are commonly reported in hypothyroidism (Panciera, 1994). However, their relative frequency in dogs with clinically similar NTI has not been reported. Therefore, the diagnostic utility of these and other routine biochemical and haematological parameters for hypothyroidism remains unclear.

Canine hypothyroidism is usually treated by oral administration of a synthetic thyroid hormone supplement. However, the dosing regime is controversial and recommendations vary among authors. Similarly, guidelines for therapeutic monitoring vary between reports and are largely empirical. Furthermore, there is a paucity of data regarding the changes which occur in biochemical and haematological parameters following the institution of therapy for hypothyroidism. Neither has the correlation of endocrine or routine clinicopathological parameters with clinical outcome been reliably studied in dogs. Consequently there are no scientifically-based objective guidelines for treating and monitoring canine hypothyroidism.

1.2 PROJECT AIMS

It was against this background that the present study was performed. The project aims were:

To fully validate commercially available total T_4, free T_4 by modified equilibrium dialysis, total T_3, cTSH, and TgAb assays for use with canine serum.

To evaluate the potential role of differential positive rate analysis and receiver operating characteristic curves in the study of diagnostic tests of canine thyroid function.

To evaluate the diagnostic utility of these assays in the investigation of dogs with suspected spontaneous hypothyroidism, and to produce guidelines for their routine use and interpretation. Furthermore, to study these tests in a population of dogs with a variety of NTI to provide additional data on the test performances in a clinical setting.

To identify clinical and clinicopathological characteristics of canine hypothyroidism and by comparison with the equivalent results from dogs with clinically similar NTI, identify the most reliable predictors of hypothyroidism. Furthermore, to identify clinical and epidemiological characteristics associated with hypothyroidism

To determine objective therapeutic guidelines for canine hypothyroidism and to evaluate the role of both endocrine tests and routine biochemical and haematological tests in monitoring dogs receiving thyroid hormone replacement therapy.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 CLINICAL MATERIAL

The initial case material comprised a series of dogs referred to the University of Glasgow Veterinary School Department of Veterinary Clinical Studies (DVCS) for investigation of clinical signs or clinicopathological abnormalities suggestive of hypothyroidism. All cases were registered with the DVCS at the time of their first consultation and assigned a unique permanent six figure hospital number. Details of the owner, patient and referring veterinary surgeon were recorded to the hospital database and updated during subsequent visits. The data recorded are listed in Appendix 1.

During the initial consultation relevant historical details were recorded in the animal's case notes including the type and duration of clinical signs, current and previous drug therapies, previous ancillary test results, and response to any previous therapies. A full physical examination was performed and the findings similarly recorded. If hypothyroidism remained a possible diagnosis, owner consent for further investigation was ascertained and the animal was hospitalised. Providing the animal had been subjected to an overnight fast, blood samples were collected by jugular venipuncture into appropriate tubes for further analyses which included all or a selection of the following; routine haematology, routine biochemistry, total T_4, endogenous cTSH, free T_4 measured by equilibrium dialysis, total T_3 and TgAb. A bovine TSH response test was then performed in each case. The result of this test was used to categorise the case as either hypothyroid or euthyroid.

Hypothyroidism was excluded in dogs with a post-TSH serum total T_4 concentration in excess of 30 nmol/L and which was at least 1.5 times greater than the basal total T_4 concentration. In these cases additional diagnostic tests were performed as required to identify the cause of disease. Hypothyroidism was confirmed in dogs with a post-TSH total T_4 concentration < 20 nmol/L. These criteria are similar to those most recently used by Peterson *et al.*, (1997) and Scott-Moncrieff *et al.*, (1998). Additional criteria were used to classify those dogs with a post-TSH total T_4 concentration which was between 20 and 30 nmol/L and which had shown an increase of at least 1.5 times the basal concentration. These additional criteria included follow-up measurement of thyroid

hormones, histopathological examination of thyroid tissue and complete recovery from a NTI without the need for thyroid hormone supplementation.

In dogs in which hypothyroidism was excluded based on the above results appropriate additional ancillary tests (urinalysis, diagnostic imaging, electrocardiography, skin scraping, dynamic adrenal function testing etc.) were performed to establish a diagnosis. These animals were usually transferred to other clinicians in the DVCS for such investigations. If thyroid status was equivocal after the initial tests, three to six month intervals were allowed elapse and the case subsequently reviewed and classified. In dogs in which hypothyroidism was confirmed treatment was instituted with L-thyroxine sodium (Soloxine, Daniels Pharmaceuticals) @ 0.02 mg/kg body weight given once daily in the morning. Owners were requested to return for repeat examinations two, six and 12 weeks after the start of treatment unless otherwise indicated. At each repeat examination the changes in clinical details were recorded and blood collected six hours after T_4 administration for total T_4 and cTSH estimation and routine biochemical and haematological analyses. The dosage of L-thyroxine was subsequently adjusted based on the clinical and clinicopathological response. If there was resolution of the clinical signs, and acceptable and stable clinicopathological results by the 12 week examination, cases were discharged from the DVCS and referred back to their primary veterinary surgeon for future monitoring. Any dogs who were not adequately controlled by the 12 week check-up continued to return to the DVCS until adequately treated.

Assessment of total T_4, free T_4, total T_3, cTSH and TgAb was also performed in serum and plasma samples collected from a series of dogs hospitalised at the DVCS with various NTI (Chapter 8). This group comprised animals being blood sampled by the attending clinician in the DVCS as part of routine diagnostic or monitoring evaluation. Dogs with a wide range of types and severity of illnesses were included but only if there was a sufficient volume of sample spare to perform subsequent thyroid hormone analyses. Clinical and historical information, and outcome of these cases was obtained by interrogation of the relevant clinician and when necessary by examination of the laboratory submission form, hospital case notes and the letters of referral. The diagnosis assigned to these cases was the one considered most likely by the attending clinician. Further details on the method of clinical information recording in these cases is provided in Chapter 8.

2.2 ROUTINE HAEMATOLOGICAL ANALYSES

Blood was collected into plastic tubes containing tri-potassium ethylenediamine tetra-acetic acid (EDTA) anticoagulant and analysed within four hours of collection using an automated MINOS-Vet haematology analyser (ABX, Montpellier). Buffered saline was used as a diluent and potassium cyanide as a lysing agent as described by the manufacturer. A blood film was prepared on a glass slide and stained with May-Grunwald-Geimsa's stain. The differential white blood cell count was performed by counting and classifying at least 200 cells using an extended battlement technique. Measured and calculated parameters are listed in Appendix 2.

2.3 ROUTINE BIOCHEMICAL ANALYSES

Blood was collected into plastic tubes containing either lithium heparin (all routine measurements except glucose) or fluoride oxalate (glucose measurement) anticoagulant and analysed within four hours of collection. The parameters measured and the analysers used are listed in Appendix 3. Quality control (QC) procedures included daily analysis of commercial QC samples including regional quality control (RQC) (Scottish Antibody Production Unit (SAPU)), Pathonorm (Biostat) and Set-Point Calibrator Universal (Bayer), as well as monthly analysis of Veterinary Sample Exchange (VSE) scheme QC material.

2.4 BOVINE THYROTROPIN RESPONSE TEST

A bovine TSH response test was carried out in the initial cases by collection of blood for total T_4 estimation immediately before and six hours after the intravenous administration of 0.1 international unit (i.u.) TSH (Thyrotropic Hormone, Sigma Chemical Company) per kg body weight (maximum dose 5 i.u. per dog). Serum total T_4 estimations were performed on each sample in the same assay run to avoid inter-assay variation.

2.5 THYROID HORMONE ANALYSES

All plain (uncoated) tubes used for hormone assays were polypropylene highly-transparent 4.5 ml tubes (Coombs-Tube, catalogue number 57.477.500, Starstedt). The pre-TSH sample was used for all hormone measurements other than the post-TSH total T_4 estimation.

Total Thyroxine

Serum total T_4 concentrations were measured using a commercial magnetic separation competitive radioimmunoassay (RIA) kit (MAGIC T_4, Chiron Diagnostics). The materials provided with the kit included one vial of rabbit anti-T_4 antibody (Ab) covalently bound to magnetic particles and suspended in phosphate buffered saline (PBS), one vial of ^{125}I-labelled T_4 tracer (less than 216 kilo Becquerels (KBq) radioactivity per vial) dissolved in PBS, and six pre-prepared T_4 standards in human serum at concentrations of approximately 0, 32, 64, 129, 193 and 386 nmol/L. All samples, standards and reagents were allowed to come to room temperature prior to use. The assay was performed according to the manufacturers instructions with the addition of 8 nmol/L and 16 nmol/L standards prepared by dilution of the supplied standards with the zero standard, to increase the working range of the assay.

Plain tubes were labelled in duplicate for standards, controls and patient samples and two tubes were labelled for total count (TC) measurement. 25 µl of either standard, control or patient serum plus 100 µl of tracer and 500 µl anti-T_4 Ab was added to each tube. All tubes were incubated at room temperature for one hour and then placed in a magnetic separation unit (MAGIC, Chiron Diagnostics) for three minutes before decanting and blotting. The remaining radioactivity was measured in counts per minute (cpm) with a gamma counter (COBRA II Auto-Gamma, Packard BioScience) and compared with those of the standards to allow estimation of the total T_4 concentration in the original patient sample.

Free Thyroxine

A modified direct dialysis kit (Free T_4 by Equilibrium Dialysis, Nichols Institute Diagnostics) was used for free T_4 estimations. The materials provided with the kit included 100 polystyrene tubes coated with anti-T_4 Ab, one vial of ^{125}I-labelled T_4 tracer (less than 270 KBq radioactivity per vial) in HEPES buffer, six standards in HEPES buffer at batch-specific concentrations of approximately 0, 3.1, 8.0, 23.2, 55.3 and 124.8 pmol/L after reconstitution with 6.0 ml deionised water, one vial of wash solution concentrate in PBS, one vial of dialysis buffer, and 40 disposable Nelson Dialysis Cells composed of an outer dialysate vial and an inner membrane cylinder. The assay was performed according to the manufacturers instructions.

Dialysate vials were labeled in singlicate for all control and patient samples. The membrane cylinder was removed, 2.4 ml dialysis buffer added to the dialysate vial and the

membrane cylinder replaced. 200 µl of patient sample or control was added to the appropriate membrane cylinders and the cells were sealed with parafilm (NESCOFILM, Nippon Shoji Kaisha Ltd.) and incubated for 16-18 hours at 37 ± 0.5°C in a water bath (SS40-A2, Grant Instruments). After incubation the membrane cylinders were immediately removed from the dialysate vials prior to RIA.

The coated tubes were labelled in duplicate for standards, control and patient sample dialysates. Two plain tubes were labelled as TC tubes and two as non-specific binding (NSB) tubes. 800 µl of standard or dialysates and 50 µl of tracer was added to appropriate tubes. These were vortexed and sealed with parafilm and incubated at 37 ± 0.5°C in a water bath (SE15, Grant Instruments). After incubation all tubes except the TC's were decanted and washed twice each with 2 ml of wash solution. After thorough draining the remaining radioactivity was measured as for total T_4. Comparison of the test sample counts with those of the standards allowed estimation of the free T_4 concentration in the original patient sample.

Total Triiodothyronine

Serum total T_3 concentrations were measured using a commercial magnetic separation competitive RIA kit (MAGIC T_3, Chiron Diagnostics). The materials provided with the kit included one vial of rabbit anti-T_3 Ab covalently bound to magnetic particles and suspended in PBS, one vial of ^{125}I-labelled T_3 tracer (less than 81 KBq radioactivity per vial) dissolved in PBS, and seven pre-prepared T_3 standards in human serum at concentrations of approximately 0, 0.38, 0.77, 1.54, 3.08, 6.16 and 12.32 nmol/L. All samples, standards and reagents were allowed to come to room temperature prior to use and the assay was performed according to the manufacturers instructions.

Plain tubes were labelled in duplicate for standards, controls and patient samples and two tubes were labelled for TC measurement. 50 µl of either standard, control or patient serum plus 100 µl of tracer and 500 µl anti-T_3 Ab was added to each tube. All tubes were incubated at room temperature for two hours and then placed in a magnetic separation unit for five minutes before decanting and blotting. After thorough draining the remaining radioactivity was measured as for total T_4. Comparison of the test sample counts with those of the standards allowed estimation of the total T_3 concentration in the original patient sample.

Thyrotropin

Endogenous serum cTSH was measured using a commercial immunoradiometric (IRMA) assay (Canine TSH IRMA, Diagnostic Products). The materials provided with the kit included 100 polystyrene tubes coated with murine anti-cTSH monoclonal Ab, two vials of ^{125}I-labelled anti-cTSH rabbit polyclonal Ab tracer (less than 270 KBq radioactivity per vial), seven cTSH standards prepared in cTSH-free canine serum at batch-specific concentrations of approximately 0, 0.15, 0.30, 0.60, 1.5, 4.0 and 12.0 ng/ml, and one vial of concentrated buffered saline solution. All samples, standards and reagents were allowed to come to room temperature prior to use. The assay was performed according to the manufacturers instructions.

The coated tubes were labelled in duplicate for standards, controls, test samples and two plain tubes were labelled for TC estimation. 100 µl of standard, control or patient serum and then 100 µl of tracer was added to all tubes prior to shaking at room temperature on a rack shaker (DPSR2, Diagnostic Products) at 200 strokes per minute for three hours. All tubes except TC's were decanted and washed twice each with 2 ml of wash solution. After thorough draining the remaining radioactivity was measured as for total T_4. Comparison of the test sample counts with those of the standards allowed estimation of the cTSH concentration in the original patient sample.

Thyroglobulin Autoantibodies

A commercial enzyme linked immunosorbent assay (ELISA) kit (Canine thyroglobulin autoantibody immunoassay kit, Oxford Laboratories) was used to assess TgAb status. The materials provided with the kit included one 96-well microtitre plate pre-coated with canine thyroglobulin (Tg), one vial of horseradish peroxidase (HRP)-conjugated rabbit anti-canine immunoglobulin G (IgG), one vial each of TgAb positive and negative reference serum, one vial of phosphate buffer, one vial of 3,3',5,5'-tetramethylbenzidine (TMB) enzyme substrate and one vial of wash buffer. The assay was performed according to the manufacturers instructions.

Samples and standards were diluted 1:100 with buffer and 100 µl was added to appropriate wells and incubated at room temperature for at least two hours. All test samples were measured in duplicate and standards in hexuplicate. Four wells were left empty as blanks. After incubation each well was washed three times and 100 µl of anti-canine IgG was added. After a further one hour incubation period the wells were washed three times and 200 µl enzyme substrate was added. After a 10 minute incubation period 50

µl 1N sulphuric acid stop solution was added to each well and the optical densities (o.d.) immediately read at 450 nm using a commercial plate reader (MRX Microplate Reader, Dynex Technologies (UK) Ltd.). Test sample o.d.'s less than the mean plus twice the s.d. of the negative standard were considered negative. Test sample o.d.'s greater than twice the mean value of the negative standard were considered positive. Those samples with o.d.'s between these limits were considered equivocal.

2.6 DATABASE INTERROGATION

The hospital computer system operated by the DVCS over the relevant period of this study was used to record animal details of all cases referred to the University of Glasgow Veterinary School. These data were used for the generation of the hospital control group used in Chapter 5. Data from proven hypothyroid and euthyroid groups was directly stored using standard personal computer (PC) compatible Microsoft software. The hospital system was operated from a Jarogate Sprite 386 (Jarogate) and used relational database software (Dataflex, Data Access Corporation) running on a CP/M operating system. Terminals for case data entry were located within the case reception office. Additional links via a local network allowed transfer of data between the central animal file and the Department of Veterinary Pathology which included haematology data. The central animal file itself stored relatively few details on each case, namely hospital number, animal name, gender, date of birth and date first seen. However this file contained references to other files which were used for storage of data relating to species, breed, owner, staff member and referring veterinary surgeon. Consequently accessing the central animal file directly allowed interrogation of numerous additional files with the appropriate case information. The interrogation of the system was performed by executing the "query database" function available within Dataflex. This function allowed the selection of the desired case details which could be saved in a delimited form to an external file. Each query was defined by case and temporal parameters and stored to PC in a compatible format using a commercial computerised spreadsheet programme (Microsoft Excel for Windows 95, version 7.0a, Microsoft Corporation). This allowed additional and more convenient Windows-based data interrogation at a later date.

2.7 DATA STORAGE

Microsoft Excel spreadsheets were used for storage, sorting, retrieval and transfer of clinical, haematological, biochemical and endocrinological data of the proven hypothyroid

and euthyroid groups. Data were arranged in rows on a single spreadsheet primarily in alphabetical order of the owner's name, and secondarily in order of visit date. Each dog-visit therefore resulted in the addition of another row of information containing relevant data as detailed in Appendix 4. This method of data storage facilitated retrieval and transfer to computerised statistical packages for subsequent analyses.

Data from cases used in Chapter 8 were collected by manual storage of various parameters to a blank template as shown in Appendix 5. Each hard copy was recorded and stored in numerical order as determined by the date of sample receipt. Many of the parameters such as age, breed, case number, date of sample and gender could be recorded directly from the sample submission form accompanying the relevant sample. Additional clinical information was obtained by interrogation of the attending clinician. A computerised database (Microsoft Access for Windows 95 version 7.00, Microsoft Corporation) was then used to store the data on PC. A file was produced which allowed storage of the appropriate parameters. This was interrogated by the design of appropriate query actions within the software.

2.8 DATA MANIPULATION

Diagnostic Sensitivity and Specificity

The diagnostic performance of the tests used in the investigation of hypothyroidism (Chapters 4 and 5) were evaluated in terms of their sensitivity and specificity (Table 1). Diagnostic sensitivity was defined as the probability that a test or procedure was positive when the disease was present and the specificity as the probability that the test or procedure was negative when the disease was not present (Griner, Mayewski, Mushlin & Greenland, 1981). Individuals with a result equal to the cut-off were considered diseased (Jensen, 1994).

$$\text{Diagnostic sensitivity} = \frac{\text{True positives}}{\text{True positives + false negatives}}$$

$$\text{Diagnostic specificity} = \frac{\text{True negatives}}{\text{True negatives + false positives}}$$

Table 1. Calculations used to determine diagnostic sensitivity and specificity

Production of Receiver Operating Characteristic (ROC) Curves and Calculation of the Differential Positive Rate (DPR)

The performance of the test under investigation across the range of possible cut-off values was represented graphically by generating a receiver operating characteristic (ROC) curve (Figure 1). A ROC curve is a graph which plots the sensitivity on the y-axis against 1-specificity on the x-axis throughout the range of possible test cut-off values. All ROC curves pass through the points 0,0 (sensitivity = 0, specificity = 1) and 1,1 (sensitivity = 1, specificity = 0). A perfect test (i.e. sensitivity and specificity both = 1) extends to the upper left hand corner of the graph (position 0,1) whereas a test with no ability to differentiate healthy from diseased individuals is a straight line with a slope of 1 extending from the origin (position 0,0) to the top right corner (position 1,1) of the graph. ROC curves for several diagnostic tests can be visually compared with the line located furthest towards the top left hand corner of the graph being the generally better test. However, a more objective assessment can be made by calculation of the area under the curve (W). The W of a perfect test equals 1 whereas a test in which W is not significantly different from 0.5 (a straight line from position 0,0 to 1,1) has no determinable ability to differentiate healthy from diseased individuals (Jensen, 1994). The W was calculated for all endocrine tests under investigation.

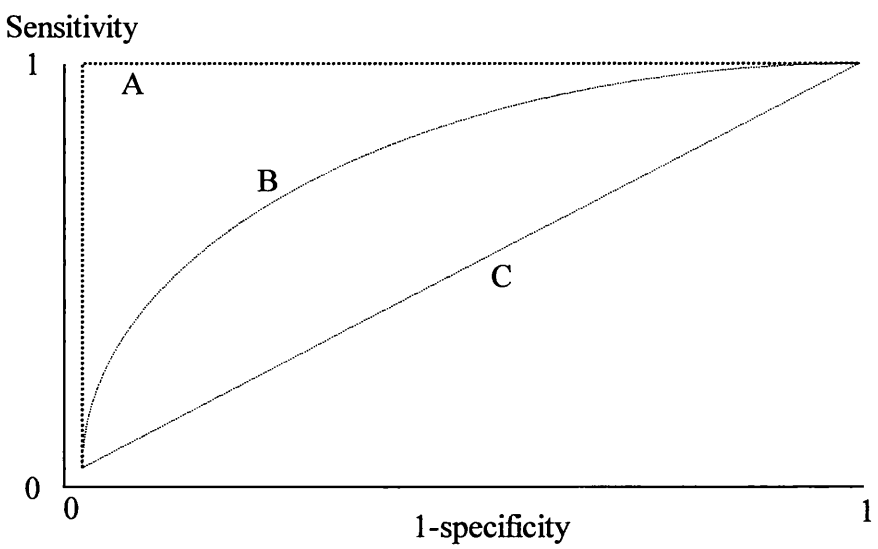


Figure 1. Representation of a Receiver Operating Characteristic (ROC) curve. Line "A" represents a diagnostically perfect hypothetical test. Line "B" represents a hypothetical test with intermediate performance characteristics. Line "C" represents a hypothetical test with no ability to differentiate affected from unaffected patients.

The cut-off value of the test associated with the greatest combined sensitivity and specificity was identified by calculation of the differential positive rate (DPR) across the range of possible cut-off values. The DPR corresponds to the sensitivity minus (1-specificity) (Ward, 1986). The computational data for DPR analysis and ROC curve construction including calculation of diagnostic sensitivity and specificity were generated by use of formulae constructed within Microsoft Excel. These are outlined in Appendix 6. ROC curve data were produced according to the method of Jensen, (1994) and then copied to a computerised graphical package (PowerPoint for Windows 95 version 7.0b, Microsoft Corporation). Graphs of the diagnostic sensitivity, specificity and DPR were also produced with Microsoft PowerPoint.

2.9 STATISTICAL ANALYSES

All statistical calculations were performed using MINITAB 9.2 for Windows (Minitab Inc.) or MedCalc Demo Version 4.16e for Windows 95 (Frank Schoonjans). Additional details of the statistical methods used are given in appropriate chapters. A probability (p) value less than 5 % ($p < 0.05$) was considered significant.

2.10 SELECTION OF CLINICAL CONTROL POPULATIONS

For the purposes of this study and particularly the investigations outlined in Chapter 5, three groups of dogs were used as the clinical control groups. Each group was chosen for a separate and specific purpose. This is discussed in detail in Chapter 5. The first control group, referred to as the euthyroid group, consisted of those dogs investigated for hypothyroidism but subsequently classified as euthyroid. This group was used to identify the characteristics which separated truly hypothyroid dogs from dogs with diseases mimicking hypothyroidism. The second control group, referred to as the hospital group, consisted of all dogs other than the hypothyroid or euthyroid groups, with complete data for age, breed and sex referred to the DVCS during the study period. These data were obtained by interrogation of the hospital computer system as previously described. If dogs were seen on more than one occasion during this interval only data from the first visit was used for statistical analyses. The hospital group was used to allow comparison of results with other studies, most of which used the equivalent cases as control populations. The third control group, referred to as the external group, consisted of data obtained from the Kennel Club (KC). These data consisted of the number of dogs of each breed first registered with the KC during the period 1994-1997 inclusive.

2.11 SELECTION OF CLINICOPATHOLOGICAL CONTROL POPULATIONS

The nomenclature used to compare the routine clinicopathological results of the hypothyroid and other dogs discussed in Chapter 5 was as recommended by Grasbeck, Siest, Wilding, Williams & Whitehead, (1979). For the purposes of this study and particularly the investigation outlined in Chapter 5, the clinicopathological results from hypothyroid dogs were compared with two reference sample groups. The first was the laboratory reference range which allowed comparison of results with the findings of previous studies. The laboratory reference range was generated by a variety of methods which included analysis of samples from "healthy" dogs, clinical experience gained from the analysis of results from dogs referred to the DVCS, and the use of broadly accepted so-called "normal" values derived from the literature. The second reference sample group was all the dogs investigated for suspected hypothyroidism but in which the diagnosis was excluded, i.e. the euthyroid group. The reference distribution was considered to be all the clinicopathological results from these cases. The reference limits were defined as the 2.5^{th} and 97.5^{th} percentiles of the reference distribution values. Results were considered abnormal if they were above or below the upper and lower reference limits, respectively.

CHAPTER 3

VALIDATION OF ASSAYS USED TO ASSESS THYROID FUNCTION

3.1 LITERATURE REVIEW

Assay validation

The reliability of an assay should be demonstrated prior to its adoption for routine use because although most QC procedures will identify changes in the performance of an analytical method, no system of QC can improve a fundamentally unsound technique (Buttner, Borth, Boutwell, Broughton & Bowyer, 1980a). The principal features which require evaluation in assay validation studies are precision, accuracy, specificity and detectability often expressed as "sensitivity" (Robinson, 1988; McLauchlan & Gowenlock, 1988). This is known as the "PASS" validation of a method. Precision includes inter-assay and intra-assay precision; accuracy is usually determined by assay of reference samples or by spiking of samples; specificity is evaluated by cross-reactivity or parallelism studies, and sensitivity can be determined using a number of methods. The PASS scheme is outlined below. In addition, consideration should also be given to the practicality of a method such as speed, cost, skills required and safety (Seneviratne, 1988).

The precision of a method is an assessment of its repeatability. This encompasses both within-run (intra-assay) precision and between-day (inter-assay) precision. The number of samples used for precision estimation is arbitrary, although 20 replicates spanning the range of clinical relevance has been suggested to be adequate (Buttner *et al.*, 1980a; Seneviratne, 1988). The mean, s.d. and the coefficient of variation (c.v.) defined as the ratio of the s.d. to the mean expressed as a percentage should be quoted. There are no standard performance criteria applicable to all assays since these vary depending on the clinical interpretation of results across the range of possible values. However, for radioimmunoassays intra-assay c.v.'s up to approximately 10 % and inter-assay c.v.'s not exceeding 15 % are often broadly acceptable (Hunter, 1978).

The accuracy of an assay is the agreement between the mean estimate of a quantity and its true value. However the true value of biological specimens is rarely known. Consequently, comparison of the method with other techniques, ideally a definitive method but more usually a reference method, is commonly used as an indicator of accuracy as is the

ability of the technique to recover a known amount of added material (Buttner *et al.*, 1980a; Robinson, 1988).

Most analytical methods show some lack of specificity commonly due to cross-reactivity with other analytes. Indeed the assessment of specificity is rather open-ended since an infinite number of molecules, drugs, food-stuffs etc. may potentially interfere with an assay. Nevertheless the use of immunoassays is generally associated with relatively high specificity due to the highly specific nature of the antigen-antibody interaction. Ideally, specificity is demonstrated by the measurement of analyte in a sample to which various structurally similar compounds have been added. Under these conditions, a highly specific assay will show no significant increase in apparent concentration whereas the concentration is likely to increase if there is significant cross-reactivity. However, in practice this can be technically awkward depending on the group of substances under test. Therefore as an alternative, the specificity of an assay can be evaluated by demonstration of linearity under dilution using a diluent known not to contain the analyte. This technique is straightforward particularly in the case of drug assay studies and therefore is widely used. However, when measuring biological entities, such as hormones, obtaining serum which is devoid of the analyte but otherwise identical to the test samples is more difficult. Collection of samples from patients with genetic deficiencies of particular hormones may be used for this purpose but clearly these are difficult to regularly obtain. Serum from the relevant species can be stripped of hormone for example by charcoal adsorption (Stockhill, 1979). However this process also results in removal of other hormones and can be complicated by the persistence of charcoal fines within the serum. An alternative method of hormone extraction from serum in which serum is passed through suspended dowex resin was reported by Salter, (1979). The addition of radiolabelled hormone to the serum prior to processing and subsequent assessment of the radioactivity remaining after extraction allows confirmation that all the hormone has been removed. This method is inexpensive and avoids the problem of charcoal fines. The use of assay "zero standards" as the diluent in parallelism studies is often used as an alternative to hormone stripped serum. A disadvantage is that the measured concentration of any analyte will usually decrease in a linear manner irrespective of the analyte being measured, as long as the diluent is free of that substance. The use of protein-free diluents such as zero standards, will also influence protein binding in the resultant samples and potentially interfere with total and particularly free hormone assays. This is particularly important in samples containing inhibitors of protein binding. This issue is discussed in more detail in Chapter 8. Therefore given all of

these, while the absence of parallelism usually indicates poor assay specificity, its presence does not guarantee it. The specificity of an assay can also be evaluated by demonstrating an appropriate biological response. For example a rise in circulating cTSH concentration following administration of the tripeptide, thyrotropin releasing hormone (TRH), or increase in total T_4 after cTSH administration would be supportive of assay specificity (Belshaw & Rijnberk, 1979). However this method is certainly not definitive. The method or methods ultimately used depends on the balance between the available resources and the purposes of the validation.

The detectability of an assay is its ability to detect small quantities of a measured component. Whilst this has no numerical value, the corresponding measured quantity is the limit of detection (LoD) which is the smallest single result that with a stated probability, usually 95 %, can be distinguished from a suitable blank (Buttner, Borth, Boutwell, Broughton & Bowyer, 1980b). Determination of the LoD allows comparison between methods, helps avoid regular misuse of an assay at values near or lower than the LoD, and recognises those results which should be reported as 'less than [LoD]'. Several methods of estimation of the LoD have been reported. The International Federation of Clinical Chemistry (IFCC) recommends repeated analysis of a suitable blank known not to contain the analyte and calculation of the LoD as the mean plus 2.6 s.d. of the blank result (Buttner *et al.,* 1980a). Alternatively, the LoD of an assay can be determined by the construction of precision profiles. This technique enables estimation of the lowest concentration of an analyte associated with an acceptable level of assay imprecision (Wilkinson, Rae, Thompson, Toft, Spencer & Beckett, 1993; Ekins & Edwards, 1997). This approach allows the varying clinical applications of assays to be accommodated without a rigid criteria being applied to all systems. Since the method relies upon the loss of precision which occurs at extremes of the standard curve it can also be used to determine the upper end of the analytical range of a method. Alternatively, the concentration of hormone producing a change in binding of a predetermined value (e.g. 2 s.d. greater than the maximal binding) has been used as corresponding to the LoD (Torres, McKeever & Johnston, 1991; Panciera & Post, 1992; Peterson *et al.,* 1997). Some workers prefer to cite the concentration of the lowest available standard as the LoD (Thoday, 1986). However, this is likely to be an unnecessarily conservative approach particularly given the sophisticated computer modeling packages now available for standard curve extrapolation (Robinson, 1988).

Validation of assays used to assess canine thyroid function

Numerous publications reporting on the laboratory or clinical performance of thyroid hormone and clinically related assays exist in the human and veterinary literature. It is outwith the scope of this thesis to attempt a review of all such reports. Therefore, a selection of those considered to be either of most historical interest, or particularly relevant to the work outlined herein were chosen for a more detailed review.

Total thyroxine

The most widely investigated diagnostic assays designed to assess canine thyroid function are total T_4 RIA's. These are usually manufactured for use with human serum but are reliable when used with canine samples because of the inter-species cross reactivity of T_4 (Feldman & Nelson, 1996). However, serum total T_4 concentration in the dog is approximately 25 % of that encountered in humans (Kaptein, Hays & Ferguson, 1994) and therefore most procedures are modified by dilution of the standards to facilitate the measurement of lower concentrations (Panciera & Post, 1992; Hall, Campbell, Chambers & Davis, 1993; Torres *et al.*, 1991).

One of the first large studies on the use of thyroid hormone RIA's in veterinary medicine was reported by Belshaw & Rijnberk, (1979). Charcoal based magnetic separation RIA's for total T_4 and total T_3 estimation in canine serum were used. The assay's were in-house methods using ^{125}I-labelled T_4 and T_3 (New England Nuclear), T_4 and T_3 rabbit antisera (Nichols Institute) and pre-supplied T_4 and T_3 standards (Sigma Chemical Co.). However only limited assay validation data were reported and the authors concentrated mainly on clinical validation of the methods.

Reimers, Cowan, Davidson & Colby, (1981) evaluated a human derived coated-tube total T_4 RIA (Autopak, Micromedic Systems) for use with canine serum and gave a full and comprehensive report on their methods and results. The assay was modified for canine serum by dilution of the lowest standard 1:2 with zero standard to improve the assay LoD. Intra-assay precision was evaluated by the repeated measurement of two samples on 10 occasions in a single assay. Inter-assay precision was evaluated by the repeated measurement of three samples in 20 consecutive assay runs. Intra-assay c.v.'s were 5.8 and 7.6 % at mean total T_4 concentrations of 8.89 and 44.27 nmol/L, respectively. Inter-assay c.v.'s were 16.4, 7.1 and 4.0 % at mean total T_4 concentrations of 8.62, 25.35 and 54.31 nmol/L, respectively. Accuracy was determined by adding known amounts (determined gravimetrically) of purified T_4 to serum samples containing previously measured

endogenous total T_4 and measuring the final concentration obtained. The recovery slope had an r value of 0.997 and an intercept of -0.17 based on analysis of three samples. However the individual results were not provided. The LoD was determined by calculation of the lower 95 % confidence limit of percentage binding of the zero standard based on 10 measurements. This was reported to be 2.19 nmol/L. Specificity was comprehensively evaluated by the production of a standard inhibition curve, assessment of cross-reactivity with chemically similar compounds and by reproducing previously observed biological responses in-vivo. The individual results of the dilutional study were not provided, but visual analysis of the inhibition curve confirmed a parallel response following dilution with the supplied standards. The relative cross-reactivity (T_4 being allocated a value of 1) with both T_3 and 3,3',5'-T_3 (reverse-T_3, rT_3) was 0.064 and was negligible with various other similar compounds. An appropriate increase in total T_4 was documented following the administration of TSH (unspecified protocol) and an appropriate decrease was reported 24 hours following thyroidectomy in cats.

Peterson, Ferguson, Kintzer & Drucker, (1984) evaluated an in-house human total T_4 RIA for use with canine serum. A commercial anti-T_4 antibody (Endocrine Sciences, Tazana) was used in the assay but the separation method of bound from unbound hormone was not specified. The intra-assay c.v. was 5.6 %, and the inter-assay c.v. was 5.8 %. Both estimates of precision were based on six samples but the mean concentrations of these were not provided. Mean ± standard error of the mean (s.e.m.) recovery of hormone added to hormone-free (charcoal stripped) serum was 98.1 ± 6.8 % (n=6), 102 ± 3.3 % (n=6), 94.8 ± 2.9 % (n=6) and 89.8 ± 1.9 % (n=6) at mean concentrations of 10.04, 20.08, 40.15 and 80.44 nmol/L, respectively, giving an average of 96.2 ± 2.2 %. However, the method used to confirm the quantity of hormone added to each sample was not provided. Parallelism was confirmed by the measurement of total T_4 in 75, 50 and 25 % dilutions of a sample with charcoal stripped serum. The resulting mean ± s.e.m. serum concentrations were 71.3 ± 1.4 % (n=6), 50 ± 2.5 % (n=7), and 21.6 ± 0.8 % (n=6) of the undiluted sample concentration, respectively. Assessment of the specificity of the assay was based on the manufacturers data which reported cross-reactivity with total T_3 and rT_3 as 0.3 and 2.0 % respectively. The LoD was reported to be 2.57 nmol/L although the method of calculation was not provided. The upper end of the working range was reported to be 643.5 nmol/L.

Panciera & Post, (1992) evaluated a commercially available coated tube human total T_4 RIA (Coat-A-Count total T_4, Diagnostic Products) for use with canine serum. The assay was performed according to the manufacturers instructions other than an additional

standard was prepared by 1:1 dilution of the lowest supplied standard with the zero standard to increase the working range. Intra-assay precision was determined by the repeated measurement of low, normal and high thyroid hormone pools each on 10 occasions within a single assay. The low pool was collected from hypothyroid dogs and the high pool was obtained from dogs following the administration of TSH. Intra-assay c.v.'s were 9.1, 8.5 and 10.0 % at mean concentrations of 10.8, 30.9 and 80.9 nmol/L, respectively. The inter-assay c.v. was reported to be 14 %. However no information regarding the method of calculation or the concentration at which it was estimated was provided. Accuracy was determined by recovery of added hormone to the low hormone pool. Following the addition of 12.87, 51.48 and 128.7 nmol/L T_4, 103, 96 and 95 % of the respective expected concentrations were measured. The LoD of the assay was calculated as the point of 90 % binding on the standard curve and reported to be 0.6 nmol/L. Specificity was assessed by dilution of the high pool with the zero standard supplied with the kit. In 1:2, 1:4 and 1:8 dilutions, 97, 77 and 72 % of the respective expected concentrations were measured. No reference was made by the authors of this paper to the apparent lack of parallelism in the dilutional study or the estimated LoD which is considerably lower than in most similar studies.

Recently, Peterson *et al.,* (1997) evaluated a widely used canine coated tube total T_4 RIA (Coat-A-Count Canine T_4, Diagnostic Products). The assay is specifically manufactured for use with canine serum by inclusion of calibrators produced with canine serum to minimise variation in protein binding between species. The assay is also optimised for use with canine samples, to facilitate the measurement of the lower thyroid hormone concentrations in this species. Intra-assay precision was determined by assaying six replicates of four pooled canine serum samples, and inter-assay precision was determined by assaying four pooled samples on four consecutive days. In each case the pooled samples ranged from low to slightly high total T_4 concentrations but actual values were not provided. Surprisingly, only a single result for both the intra-assay c.v. (8.0 %) and inter-assay c.v. (5.0 %) was actually reported. Whilst the description of the method of estimating precision was therefore rather ambiguous it is likely that the quoted values represented the mean c.v. of the four hormone pools. This is an unsatisfactory method of reporting validation data because the mean precision value may lie within acceptable limits despite unacceptably poor c.v.'s at various points of clinical importance on the standard curve. Accuracy was not assessed. The LoD was defined as the apparent concentration of total T_4 that produced a change in binding two s.d. greater than that of the zero standard and was

reported to be 2.5 nmol/L. Specificity was evaluated by dilution of a pooled sample of high total T_4 concentration. Parallelism of the standard curve was reported but the data were not provided.

Free thyroxine

The issue of free T_4 estimation is controversial and is discussed more fully in Chapter 4. Only methodologies which are considered to be genuinely free hormone techniques will be reviewed. All free T_4 assays are manufactured primarily for use with human samples. However these are usually considered to be applicable for use with canine samples.

Ferguson & Peterson, (1992) reported the use of a modified equilibrium dialysis technique for free T_4 measurement in canine serum based on an original human study of Sterling & Brenner, (1966). In brief, this method involved the addition of predialysed ^{125}I-T_4 in bovine serum albumin (BSA) to undiluted canine serum. The serum was then dialysed against a standard buffer for 16-20 hours. Following dialysis, the tracer was precipitated with a magnesium precipitation solution and separated by repeated centrifugation and washing of the precipitate. The free T_4 fraction was then determined and the free T_4 concentration calculated as the total T_4 concentration multiplied by the free T_4 fraction. The intra-assay and inter-assay c.v.'s reported were 8.2 and 16.6 %, respectively. However, no information was provided regarding the method of estimation of the assay precision or the mean free T_4 concentrations at which they were calculated. This method was time consuming and technically awkward and was not considered to be of value in a routine clinical setting.

Nelson & Tomei, (1988) designed a re-usable dialysis cell and a complex buffer which allowed dialysis of human serum samples. Free T_4 concentration was then directly measured on the dialysate using an ultrasensitive T_4 RIA. Intra-assay precision was assessed by dialysis of a single serum pool in 57 cells. The intra-assay c.v. was 5.3 % at a mean free T_4 concentration of 19.30 pmol/L. Inter-assay precision was assessed by dialysis of a separate single pooled serum sample on 57 different days over a four month period. The dialysed sample inter-assay c.v. was 6.9 % at a mean free T_4 concentration of 23.17 pmol/L. The inter-assay precision of the RIA was also evaluated on the same 57 days by repeated RIA measurement of a separate pooled dialysate. The dialysate inter-assay c.v. was 7.4 % at a mean free T_4 concentration of 18.02 pmol/L. The variability was similar for both the serum pool (subjected to dialysis followed by RIA on each of the 57 occasions) and the dialysate pool (subjected to only RIA on each of the 57 occasions) suggesting that

the dialysis step added little to the variability in results. No data on assay accuracy or specificity of the RIA was supplied. However, the normal recovery test used for assessing accuracy cannot be applied to the determination of free T_4 prior to dialysis, because the free T_4 concentration depends both on the quantity of T_4 added and on the affinity and concentration of binding proteins present (Helenius & Liewendahl, 1983). The putative working range of the assay defined as the free T_4 concentration corresponding to 90 % (LoD) and 13 % (upper limit) of the binding of the zero standard was 2.57 to 164.74 pmol/L, respectively. Additional validation work included an assessment of the effect of repeated freeze-thaw cycles and prolonged storage of frozen samples on measured free T_4 concentration. A pooled sample frozen for over three months (temperature not provided) showed no significant alteration in free T_4 concentration. Similarly, 10 repeated freeze-thaw cycles did not result in any significant change in free T_4 concentration. This method was subsequently developed into a commercially applicable kit form suitable for routine laboratory use (Free T_4 by Equilibrium Dialysis, Nichols Institute Diagnostics).

Panciera & Post, (1992) evaluated the above equilibrium dialysis kit for use with canine serum. Intra-assay precision was determined by the repeated measurement of free T_4 in three serum pools collected from hypothyroid dogs (low pool), healthy dogs (medium pool) and following administration of exogenous TSH to healthy dogs (high pool) each on 10 occasions. Intra-assay c.v.'s were reported to be 19.7, 7.3 and 10.8 % at mean concentrations of 0.57, 1.89 and 4.27 pmol/L, respectively. However the mean values reported are exceptionally low compared to all other similar human or veterinary reports and were almost certainly misquoted. It is possible that the values actually correspond to conventional units (ng/dl) rather than Système International d'Unités (SI) units which are considered preferable. Assuming this to be the case, the mean free T_4 concentrations in the low, normal and high pools were most likely 7.34, 24.32 and 54.95 pmol/L, respectively. These figures would be much more in keeping with other published values. The inter-assay c.v. was reported to be 17 %. However no indication of the free T_4 concentration to which this value refers, or the method of calculation was provided. Accuracy of the RIA was assessed by the addition of T_4 standards (supplied with the kit) to the dialysate. Recovery rates were 91, 120 and 111 % of the respective expected concentrations at increasing concentrations of added T_4. The assay LoD was defined as the point of 90 % total binding on the standard curve and reported to be 0.24 pmol/L respectively. Again, it is likely that the units were misquoted and the true value was therefore probably 3.09 pmol/L. Assay specificity was assessed by dilution of the high thyroid hormone serum pool in the ratios

1:2, 1:4 and 1:8 with the zero standard supplied with the kit. However, 122, 119, 145 and 115 % of the respective expected concentrations were reported as the results but the actual data were not provided. It is unclear from the manuscript, but appears that the serum samples were diluted then dialysed. There was no explanation provided to justify either the publication of four results from only three dilutions, or the particularly elevated value of 145 %. Once again it seems likely that problems in production of the manuscript of that study were at least in part responsible for the confusing data published.

Peterson *et al.*, (1997) evaluated the same commercial dialysis free T_4 methodology as Panciera & Post, (1992) and determined intra-assay precision by repeated measurement of four pooled serum samples ranging from low to slightly high free T_4 concentration six times within a single assay. The inter-assay precision was determined by repeated measurement of the same four pooled samples on four separate days. The intra-assay and inter-assay c.v.'s were reported to be 11.1 and 9.5 %, respectively. As was the case for the total T_4 assay validation results published in that study, these figures are assumed to be an average of the c.v.'s from all the pooled samples. Once again, this is an unsatisfactory method of result reporting. No assessment of assay accuracy was made. The LoD, defined as the concentration of free T_4 that produced a change in binding two s.d. greater than that of the negative control samples, was 2.0 pmol/L. Specificity was demonstrated by serial dilution of a high free T_4 concentration serum sample with the assay zero standard. The curve produced was reported to be parallel to the standard curve but the data were not provided. Although it is not completely clear from the manuscript, the authors apparently diluted serum samples then performed the dialysis. Assuming this to be the case, there is no explanation offered by the authors to explain a parallel free T_4 response to the standard curve. As discussed in chapter 4, this would not be expected of a true free hormone assay. As with the report by Panciera & Post, (1992), the poor description of the precise technique used for the assay validation, makes interpretation of the results and author's conclusions more difficult.

Total triiodothyronine

Reimers, Mummery, McCann, Cowan & Concannon, (1984) evaluated a human derived coated-tube total T_3 RIA for use with canine serum. Intra-assay precision was evaluated by the repeated measurement of two samples on 10 occasions in a single assay. Inter-assay precision was evaluated by the repeated measurement of three samples in 20 consecutive assay runs. Intra-assay c.v.'s were 12.8 and 7.6 % at mean total T_3 concentrations of 0.72

and 2.03 nmol/L, respectively. Inter-assay c.v.'s were 12.2, 14.0 and 11.6 % at mean total T_3 concentrations of 1.26, 1.65 and 2.66 nmol/L, respectively. Accuracy was determined by adding known amounts (determined gravimetrically) of purified T_3 to serum samples containing endogenous total T_3 and measuring the final concentration obtained. The recovery slope had an r value of 0.993 and an intercept of -0.19 based on analysis of three samples. However the individual results were not provided. The LoD was determined by calculation of the lower 95 % confidence limit of percentage binding of the zero standard based on 10 measurements. This was reported to be 0.22 nmol/L. Specificity was evaluated by the production of a standard inhibition curve and assessment of cross-reactivity with chemically similar compounds. The individual results of the dilutional study were not provided, but visual analysis of the inhibition curve confirmed a parallel response following dilution with the supplied standards. The relative cross-reactivity (T_3 being allocated a value of 1) was 0.0013 with total T_4, 0.0019 with 3,5-diiodothyronine (3,5-T_2), 0.0001 with rT_3 and was negligible with various other similar compounds.

Peterson *et al.*, (1984) evaluated an in-house total T_3 RIA for use with canine serum using a method designed by Lieblich & Utiger, (1972). Anti-T_3 Ab was prepared by repeated immunisation of rabbits with T_3-BSA conjugate. Commercially available radiolabelled T_3 (Sigma Chemical Co.) was used and separation of bound from unbound hormone was achieved by precipitation using goat anti-rabbit IgG followed by centrifugation. The intra-assay c.v. was 6.6 %, and the inter-assay c.v. was 13.4 %. Both estimates of precision were based on repeated measurement of five samples but the mean concentrations of these were not provided. Mean (± s.e.m.) recovery of hormone added to stripped serum was 113.2 ± 4.9 % (n=4), 97.7 ± 7.8 % (n=4), 110.3 ± 2.0 % (n=4) and 101.7 ± 4.4 % (n=3) at mean total T_3 concentrations of 0.48, 0.96, 1.92 and 3.85 nmol/L, respectively. However, the method used to confirm the quantity of hormone added to each sample was not provided. Parallelism was confirmed by the measurement of total T_3 in 75, 50 and 25 % dilutions of a sample with stripped serum. The resulting mean (± s.e.m.) serum concentrations were 78.8 ± 3.0 % (n=6), 55.8 ± 2.3 % (n=6) and 24.7 ± 1.0 % (n=5) of the undiluted sample, respectively. The LoD was reported to be 0.12 nmol/L although the method of determination was not provided. The upper end of the working range was reported to be 7.7 nmol/L.

Panciera & Post, (1992) evaluated a commercially available coated tube human total T_3 RIA for use with canine samples. Intra-assay precision was determined by the repeated measurement of low, normal and high thyroid hormone pools each on 10

occasions within a single assay. The serum pools were collected as described for total T_4. Intra-assay c.v.'s were 11.1, 9.9 and 6.5 % at mean total T_3 concentrations of 0.49, 1.76 and 2.83 nmol/L, respectively. The inter-assay c.v. was reported to be 11 %. However, no information regarding the method of calculation or the concentration at which it was estimated was provided. Accuracy was determined by recovery of added hormone (using supplied kit standards) to the low hormone pool. Following addition of approximately 0.31, 0.77 and 1.54 nmol/L T_3, 92, 90 and 97 % of the respective expected concentrations were measured. The LoD of the assay was calculated as the point of 90 % binding on the standard curve and reported to be 0.26 nmol/L. Specificity was assessed by dilution of the high pool with the zero standard supplied with the kit. In 1:2, 1:4 and 1:8 dilutions, 107, 109 and 114 % of the respective expected concentrations were measured.

Recently, Peterson *et al.*, (1997) evaluated a coated tube total T_3 RIA designed for use with canine serum (Coat-A-Count Canine T_3, Diagnostic Products). The assay is specifically manufactured for use with canine serum by inclusion of calibrators produced with canine serum to minimise variation in protein binding between species. The assay is also optimised for use with canine samples. Intra-assay and inter-assay precision were determined using the same protocol as described for total T_4 in the same study. The intra-assay and inter-assay c.v.'s were reported to be 6.2 and 3.4 %, respectively. However the ambiguities and assumptions discussed earlier remain applicable. Accuracy was not assessed in this study. The LoD was defined as the apparent concentration of total T_3 that produced a change in binding two s.d. greater than that of the zero standard. The LoD was reported to be 0.2 nmol/L. Specificity was evaluated by dilution of a pooled sample of high total T_3 concentration. A parallel curve was reported but the data were not provided.

Thyrotropin

The measurement of endogenous serum cTSH has been attempted for nearly 20 years. Until recently, like total T_4 and total T_3 methods, most reports concerned the use of human assays. However, unlike the iodothyronines, the cross reactivity of the human and canine TSH molecules, most probably the β-subunit, was inadequate and consequently these assays were of little diagnostic value in the dog (Larsson, 1981). Similarly, early assays purporting to be canine-specific were of little clinical value. The cTSH assay produced by Quinlan & Michaelson, (1981) was unable to detect cTSH concentrations less than 1.25 ng/ml which is inadequate for routine use. The assay evaluated by Rachofsky, Chester & Hightower, (1988) had a LoD of 1.5 ng/ml and the results from dogs investigated for

hypothyroidism were no more predictive of a favourable response to thyroid hormone replacement therapy (THRT) than total T_4 estimations alone. More recently a new range of commercial cTSH kit assays have been released. The three methodologies currently available are IRMA, chemiluminescent and ELISA techniques. The methods were all released simultaneously by the manufacturer and differ only in the signalling method used, the cTSH antibody being equivalent between methods. A review of the reports of validation of these assays is provided.

Williams, Scott-Moncrieff, Bruner, Sustarsic, Panosian-Sahakian, Unver & Said El Shami, (1996) reported the results of validation of the IRMA assay for cTSH. Intra-assay and inter-assay precision were assessed by measurement of cTSH in three serum samples each on 20 occasions within a single assay, and in 20 different assays respectively. The intra-assay c.v. was 3.8, 2.3 and 2.4 % at mean cTSH concentrations of 0.26, 0.88 and 4.6 ng/ml, respectively. The inter-assay c.v. was 3.8, 3.3 and 2.1 % at mean cTSH concentrations of 0.26, 0.92 and 4.7 ng/ml, respectively. Accuracy was assessed by the addition of three different solutions of cTSH (9.8, 39.0 and 78.0 ng/ml) to each of three serum samples in the ratio 19:1. The method of calculation of the cTSH concentrations in these spiking solutions was not reported, but presumably standard solutions used for assay development were employed. Measured cTSH values in spiked samples were between 95 and 110 % of the expected concentrations. The LoD was defined as the cTSH concentrations that produced a change in binding two s.d. greater than that of the zero standard. The upper working limit was defined as the cTSH concentration that produced a change in binding greater than two s.d. from the maximum observed. The working range of the assay was reported as 0.01 to 12.0 ng/ml. Assay specificity was assessed by dilution of three samples 1:1, 1:3, 1:7 and 1:15 with the zero calibrant. Observed cTSH concentrations in diluted samples were between 97 and 109 % of the expected values. Specificity of the assay was also inferred from the biological response in six beagle dogs in which hypothyroidism was induced by radiothyroidectomy, and subsequently treated with standard THRT. Confirmation of thyroid status was achieved by estimation of total T_4 concentrations before and after exogenous TSH administration. Circulating cTSH concentrations increased by approximately 35 fold 21 days after induction of hypothyroidism and returned to values not significantly different to the original concentrations within 14 days after starting THRT. The mean cTSH concentrations were approximately 0.1 ng/ml prior to radiothyroidectomy, peaking at approximately 4 ng/ml 28

days after radiothyroidectomy, and decreasing to less than 1 ng/ml after institution of THRT. However, the actual data were not provided other than in graphical form.

Jensen *et al.,* (1996) also validated the cTSH IRMA assay although in considerably less detail. Intra-assay and inter-assay c.v.'s were reported to be 10.6 and 9.7 %, at cTSH concentrations between 0.05 to 0.85 ng/ml, respectively. However, the protocol was not provided. Assay accuracy was evaluated by the addition of 75 µl of each of the calibrators into 425 µl aliquots of a serum pool with a measured cTSH concentration of 0.15 ng/ml. This left the serum matrix relatively unaffected, and cTSH was recovered in a linear and proportional manner. The assay LoD was not independently assessed. Analytical specificity was evaluated by the serial dilution of two samples with normal (0.07 ng/ml) and elevated (0.85 ng/ml) cTSH concentrations, using the zero standard as the diluent. The curve obtained was not parallel to that of the standard curve and the authors suggested that this may have been related to differences in matrix composition between the canine samples and the zero calibrant. The authors reported that there was no significant difference in cTSH concentration in paired samples of serum or heparinised plasma from both normal and hypothyroid dogs, although the data were not provided.

Meij, Mol & Rijnberk, (1996) evaluated the same cTSH IRMA assay and reported intra-assay and inter-assay c.v.'s of 2.4 and 3.9 %, at mean cTSH concentrations of 4.6 and 3.6 ng/ml, respectively. No assessment of accuracy was made. The LoD was reported to be 0.03 ng/ml although the protocol for estimation was not provided. Negligible cross reactivity with canine leutenising hormone (LH) and follicle stimulating hormone (FSH) was reported but the supporting data were not provided.

Peterson *et al.,* (1997) evaluated the same cTSH IRMA assay for use with canine serum. Intra-assay and inter-assay precision were determined using the same protocol as described for total T_4 in the same paper. The intra-assay and inter-assay c.v.'s were reported to be 4.7 and 2.5 %, respectively. As described under total T_4 and total T_3 the ambiguities and assumptions discussed earlier apply. Accuracy was not assessed in this study. The LoD was defined as the apparent concentration of cTSH that produced a change in binding two s.d. greater than that of the zero standard. The LoD was reported to be 0.01 ng/ml. Specificity was evaluated by dilution of a pooled sample of high cTSH concentration. A parallel curve was reported but the data were not provided.

Scott-Moncrieff *et al.,* (1998) evaluated the chemiluminescent enzyme immunoassay for cTSH. Intra-assay c.v.'s were reported to be 5.9, 3 and 6.8 % at mean cTSH concentrations of 0.07, 0.13 and 1.80 ng/ml, respectively. Inter-assay c.v.'s were

reported to be 7.0 and 4.8 % at mean cTSH concentrations of 0.30 and 2.90 ng/ml, respectively. However, no details of the number of samples or protocols for the precision studies were provided. No accuracy data were provided. The LoD was reported to be 0.05 ng/ml but the method of estimation was not provided. A clinical reference range was reported as less than 0.65 ng/ml based on analysis of samples from 62 healthy dogs. However, no additional information on the method of calculation was provided.

Ramsey, Evans & Herrtage, (1997) evaluated the ELISA method for cTSH estimation. Intra-assay precision was evaluated by testing two samples 10 times on two separate occasions. The intra-assay c.v. was reported to be less than 5 % but the actual value was not provided. Inter-assay precision was evaluated by repeated estimation of control samples in different assays. The inter-assay c.v. was reported to be 14, 18, 8.0 and 5.0 % at mean cTSH concentrations of 0.17, 0.29, 1.24 and 2.67 ng/ml, respectively. However the number of assays over which the precision was evaluated was not specified. Accuracy was not assessed. The LoD was determined by estimation of cTSH in 10 blank samples and reported to be 0.01 ng/ml. However the method of calculation was not provided. Assay specificity was not evaluated. The inter-assay c.v's reported for reference range cTSH concentrations were markedly higher in this study than in any other report of cTSH assay validation. The difference is presumably related to the different signalling technique between the methods and indicate that the IRMA (Williams *et al.,* 1996; Jensen *et al.,* 1996; Meij *et al.,* 1996; Peterson *et al.,* 1997) and chemiluminescent (Scott-Moncrieff *et al.,* 1998) techniques are considerably more reproducible than the ELISA method.

Thyroglobulin autoantibodies

Nachreiner *et al.,* (1998) evaluated a commercially available ELISA for canine TgAb estimation (Canine thyroglobulin autoantibody immunoassay kit, Oxford Laboratories). All o.d. results were expressed as a percentage of negative control sample. Unfortunately, the description of the method of determination of precision was unclear. Apparently, the intra-assay c.v. was calculated by the repeated analysis of two replicates at the beginning and end of eight assay runs. The intra-assay c.v. from each run was calculated and then the average figure for all runs subsequently determined. Using this approach, the intra-assay c.v. was reported to be 7.6 %. This approach is unusual. The inter-assay c.v. was calculated as the variation in a single sample o.d. over 10 consecutive assay runs when expressed as a percentage of the positive control. The inter-assay c.v. was reported to be 26.5 %.

Quantitative evaluation of assay accuracy is more difficult with this type of methodology since the results are reported as an o.d. relative to a qualitatively defined standard sample to which an absolute concentration of TgAb is not assigned. The LoD of the assay was not evaluated. Given the quantitative nature of the results, and the method of sample result categorisation (i.e. based on relative o.d. compared to the negative control) the assay is less amenable to this type of analysis and the absence of a standard curve makes the differentiation of the LoD from a zero result more difficult. In addition the clinical utility of this assay is such that the differentiation of low from negative results is of no practical relevance. This is presumably why the LoD was not determined. Assay specificity was functionally evaluated by comparison of thyroid histopathology from eight dogs with, and 10 dogs without, a positive TgAb result. All TgAb positive cases had evidence of mild to diffuse lymphocytic thyroiditis whereas all dogs negative for the autoantibody had no evidence of thyroiditis. Specificity was also functionally evaluated by the measurement of TgAb in serum from 146 dogs with a variety of non-thyroidal diseases. Eight (5 %) cases were TgAb positive confirming a relatively high functional specificity of the assay.

Iverson, Jensen, Hoier, Skydsgaard & Kristensen, (1998) developed and validated a separate TgAb ELISA from that reported by Nachreiner *et al.,* (1998). In each assay run, samples from a single dog with hypothyroidism and lymphocytic thyroiditis, and two dogs without hypothyroidism or thyroiditis were measured in quadruplicate. A "K" value was calculated for each run, defined as the difference between the o.d. from the hypothyroid sample and the mean of the two normal samples. Each test sample was expressed as an "Ab score". The Ab score was defined as the test sample o.d. expressed as a percentage of the K value. Intra-assay precision was evaluated by analysis of three samples (low, medium and high Ab scores) at least 15 times in a single assay run. Inter-assay precision was evaluated by repeated measurement of the samples from the control dogs in 15 different assay runs. Intra-assay c.v.'s were 3.2, 4.9 and 2.0 % in the low, medium and high Ab score samples, respectively. Inter-assay c.v's were 4.6, 7.3 and 9.9 % for the corresponding samples, respectively. Accuracy was not evaluated. The LoD was calculated as the zero value (NSB) plus two s.d. of the results of 13 duplicate analyses from samples with an Ab score less than 7.5 %. The LoD was reported to be 5.6 %. Assay specificity was comprehensively evaluated by estimation of TgAb in eight samples from TgAb positive dogs, before and following immunoglobulin precipitation with polyethyleneglycol 6000 (PEG). In addition, TgAb positive samples were incubated with purified Tg and re-analysed for the autoantibody. TgAb was estimated in another series of samples before and following

incubation with buffer to which either T_3 or T_4 had been added. Samples incubated with either PEG or Tg had a significant and almost complete reduction in assay signal confirming the assay's specificity for TgAb. Samples incubated with T_3 or T_4 showed a significant but smaller reduction in TgAb result, with those incubated with T_3 showing the greatest changes. The explanation for this is less clear, but may be attributable to the presence of T_3 autoantibodies (T_3Ab) or T_4 autoantibodies (T_4Ab) in some of the serum samples. T_3Ab and T_4Ab are believed to be subsets of TgAb that recognise particular epitopes on the Tg molecule (Gaschen, Thompson, Beale & Keisling, 1993). Alternatively changes in sample matrix following the addition of the buffer medium may have been responsible. Unlike the kit evaluated by Nachreiner *et al.,* (1998), the method studied by Iverson *et al.,* (1998) has not yet been made commercially available.

3.2 INTRODUCTION

All of the kits used to assess thyroid function in this study were commercially available immunoassays and were supplied with details of the manufacturers own validation data. However, not only were some of the kits designed for human sera, but previous reports of diagnostic and validation performance of many thyroid function tests have often been incomplete and occasionally produced conflicting information. Consequently, an assessment of technical validity and reliability for each assay was considered necessary prior to diagnostic use. The principal objectives when validating each assay were as adopted by the IFCC (Buttner *et al.*, 1980a). This consisted of confirmation of their practicality for routine laboratory use and assessment of their reliability characteristics namely precision, accuracy, specificity and detectability when used with canine sera. The assessment of certain validation characteristics was not always practical and when possible these data were obtained from the available literature. Validation of the TgAb assay in particular was more limited due to the qualitative nature of the assay results as discussed.

3.3 MATERIALS AND METHODS

Sample Collection

Blood samples used for the validation studies were collected and stored from dogs referred to the DVCS as described in Chapter 2. A number of healthy dogs were also blood sampled as part of a routine health check programme. When the sample volume permitted, these samples were also stored for inclusion in the validation experiments. A number of these samples were aliquoted prior to freezing to avoid repeated freeze thaw cycles during inter-assay precision studies.

General Statistics

When calculating the LoD of each assay, raw data (cpm) were used for statistical analysis before being converted to an equivalent LoD by calculation of the mean - 2.6 s.d. (mean + 2.6 s.d. in the case of cTSH) of the blank value as described by Buttner *et al.*, (1980a). Pre and post-gonadotropin releasing hormone (GnRH) stimulation cTSH concentrations were compared using a paired t-test.

Specific Assay Validations

Total Thyroxine

Total T_4 was measured with a commercial competitive RIA (MAGIC T_4, Chiron Diagnostics) as described in Chapter 2. Intra-assay precision was determined by the repeated measurement of 10 duplicates of three samples with varying total T_4 concentration (low, intermediate and high) in a single assay run. Inter-assay precision was determined by the repeated measurement of three samples (low, intermediate and high) in duplicate in 10 consecutive assay runs. Specificity was determined by measurement of total T_4 in serial 20 % dilutions of a single sample (original concentration 21.2 nmol/L) using the zero standard supplied with the kit as the diluent. Assay cross-reactivity data were provided by the manufacturer. The LoD was determined by repeated measurement of total T_4 in 20 replicates of the zero standard supplied with the kit.

Free Thyroxine

Free T_4 was measured using a commercial modified dialysis technique (Free T_4 by Equilibrium Dialysis, Nichols Institute Diagnostics) as described in Chapter 2. Intra-assay precision was determined by the repeated measurement of 10 duplicates of three samples with varying free T_4 concentration (low, intermediate and high) in a single assay run. Inter-assay precision was determined by the repeated measurement of three samples (low, intermediate and high) in duplicate in six consecutive assay runs. Specificity was determined by measurement of free T_4 in serial dilutions of a single dialysate using the zero standard supplied with the kit as the diluent. Assay cross-reactivity data were provided by the manufacturer. The LoD was determined by repeated measurement of free T_4 in 20 replicates of the zero standard supplied with the kit.

Total Triiodothyronine

Total T_3 was measured with a commercial competitive RIA (MAGIC T_3, Chiron Diagnostics) as described in Chapter 2. Intra-assay precision was determined by the repeated measurement of 10 duplicates of three samples with varying total T_3 concentration (low, intermediate and high) in a single assay run. Inter-assay precision was determined by the repeated measurement of three samples (low, intermediate and high) in duplicate in four consecutive assay runs. Specificity was determined by measurement of total T_3 in serial dilutions of a single sample using the zero standard supplied with the kit as the diluent. Assay accuracy and cross-reactivity data were provided by the manufacturer. The LoD was

determined by repeated measurement of total T_3 in 20 replicates of the zero standard supplied with the kit.

Thyrotropin

Circulating cTSH was measured with a commercial assay (Canine TSH IRMA, Diagnostic Products) as described in Chapter 2. Intra-assay precision was determined by the repeated measurement of 10 duplicates of three samples with varying cTSH concentration (low, intermediate and high) in a single assay run. Inter-assay precision was determined by the repeated measurement of three samples (low, intermediate and high) in duplicate in 10 consecutive assay runs. Specificity was determined by measurement of cTSH in serial dilutions of a single sample using the zero standard supplied with the kit as the diluent. Cross reactivity with similar molecules (LH and FSH) was assessed by measurement of cTSH in sera collected from three entire female dogs prior to and 60 minutes following the intravenous administration of 0.32 μg GnRH analogue (Receptal, Hoechst Roussel Vet). Assay accuracy data were provided by the manufacturer. The LoD was determined by repeated measurement of cTSH in 20 replicates of the zero standard supplied with the kit.

Thyroglobulin Autoantibody

TgAb was measured with a commercial assay (Canine thyroglobulin autoantibody immunoassay, Oxford Laboratories) as described in Chapter 2. Intra-assay precision was assessed by the repeated measurement of 20 replicates of each of two samples with either negative or positive TgAb status. Inter-assay precision was assessed by the repeated measurement in duplicate of each of three samples with negative, equivocal and positive TgAb status in six consecutive assay runs. Precision was assessed by both quantitative and qualitative methods. Quantitative assessment of the validation samples was performed by determination of the c.v.'s from each o.d. when expressed as a percentage of the negative control value. Qualitative assessment of the results was made by examination of the precision with which validation samples were correctly classified as either positive, negative or equivocal. Classification of samples was as recommended by the kit manufacturer as described in Chapter 2.

3.4 RESULTS

Total Thyroxine

Intra-assay precision results are summarised in Appendix 7. Inter-assay precision results are summarised in Appendix 8. Linearity under dilution results are summarised in Appendix 9. Results of LoD determination are summarised in Appendix 10. Cross reactivity of the assay was 2.7 % with tetraiodothyroacetic acid, 4.5 % with L-triiodothyronine and 0.11 % with D-triiodothyronine.

Free Thyroxine

Intra-assay precision results are summarised in Appendix 11. Inter-assay precision results are summarised in Appendix 12. Linearity under dilution results are summarised in Appendix 13. Results of LoD determination are summarised in Appendix 14. Cross-reactivity was less than 0.05 % with each of D-thyroxine, T_3 and rT_3.

Total Triiodothyronine

Intra-assay precision results are summarised in Appendix 15. Inter-assay precision results are summarised in Appendix 16. Linearity under dilution results are summarised in Appendix 17. Results of LoD determination are summarised in Appendix 18. Cross reactivity of the assay was 0.2 % with tetraiodothyroacetic acid, 0.14 % with D-thyroxine and 0.12 % with L-thyroxine. Mean recovery of T_3 from spiked samples was 103.1 % with a range of 96.3 to 118.3 %.

Thyrotropin

Intra-assay precision results are summarised in Appendix 19. Inter-assay precision results are summarised in Appendix 20. Linearity under dilution results are summarised in Appendix 21. Results of LoD determination are summarised in Appendix 22. Serum cTSH concentration (mean ± s.d.) was 0.13 ± 0.01 ng/ml and 0.14 ± 0.07 ng/ml prior to and following GnRH administration, respectively. There was no significant difference between pre and post-GnRH results suggesting minimal cross reactivity with LH and FSH. Recovery of cTSH from spiked samples varied from 95 to 110 % of expected values.

Thyroglobulin Autoantibodies

Quantitative intra-assay precision results are summarised in Appendix 23. Quantitative inter-assay precision results are summarised in Appendix 24. Qualitative intra-assay

precision results are summarised in Appendix 25. Qualitative inter-assay precision results are summarised in Appendix 26.

3.5 DISCUSSION

The results of the validation studies were generally considered good to excellent. Each of the methods was practical and with the exception of the free T_4 assay could be comfortably performed in a single day.

The total T_4 assay demonstrated good intra- and inter-assay precision even at low values despite extrapolation of the standard curve downwards by dilution of the standards. This is similar to the findings of previous studies (Reimers *et al.,* 1981; Peterson *et al.,* 1984; 1997). Data on the accuracy of the methods was obtained from the available literature. This avoided the need for hormone recovery experiments which were considered either excessively demanding for the purposes of the current study (total T_4 and T_3) or impractical or impossible given the nature of the analytes being estimated (free T_4, cTSH and TgAb). In addition, previous reports have clearly demonstrated that human total T_4 assays are usually highly accurate when used with canine serum (Reimers *et al.,* 1981, Peterson *et al.,* 1984) The LoD was 4.7 nmol/L which is also similar to that of previous studies (Reimers *et al.,* 1981; Eckersall, McEwan & Mooney, 1991; Peterson *et al.,* 1997). Specificity was considered adequate with total T_4 results between 104 and 112 % of the expected values after dilution. These results lie somewhere in-between those reported by Panciera, MacEwan, Atkins, Bosu, Refsal & Nachreiner, (1989) and Panciera & Post, (1992).

The free T_4 kit was the most time consuming and expensive of the assays examined. The overnight dialysis incubation required considerable preparation and practically was more awkward than the other methods. However, the method was not considered prohibitively inconvenient and could be employed within a veterinary laboratory with the appropriate facilities. Of particular importance in the performance of the free T_4 assay was the overnight incubation temperature which had to be accurately controlled. The precision studies were considered adequate and although an intra-assay c.v. of 15.7 % at 10.4 pmol/L is just outwith the guidelines of Hunter, (1978) it was considered adequate given the two steps involved. Because of the differences in methodologies for free T_4 estimation the results of this study should only be compared with reports of equivalent methods. Precision results were similar to previous studies (Nelson & Tomei, 1988; Ferguson &

Peterson, 1992; Panciera & Post, 1992; Peterson *et al.*, 1997) and the LoD was similar to that reported by Nelson & Tomei, (1988) and Peterson *et al.*, (1997) despite the different methods of calculation. However, the LoD reported by Panciera & Post, (1992) was less than 12 % of that found in both the present study and that of Peterson *et al.*, (1997). Various errors were present in that paper and the apparent differences in LoD are probably simply the result of the publication of incorrect values as described previously.

The total T_3 assay validation results were considered to be excellent. The intra- and inter-assay precision results were under 8 % at all concentrations of total T_3. This is in agreement with previous studies (Kemppainen *et al.*, 1983; Larsson, 1988; Panciera & Post, 1992; Peterson *et al.*, 1997). The LoD was 0.14 nmol/L which is very similar to other reports despite different methods of calculation (Hall *et al.*, 1993; Li, Chen, Tiller & Kunkle, 1986; Reimers, Lawler, Sutaria, Correa & Erb, 1990; Peterson *et al.*, 1997). Specificity results were good with 94 to 112 % of the expected concentrations being measured following dilution. This is in agreement with Peterson *et al.*, (1997).

The present study found intra-assay precision of the cTSH assay to be excellent across the range of clinical relevance supporting the findings of Williams *et al.*, (1996). The inter-assay precision was adequate at mean concentrations of 0.40 ng/ml and 2.17 ng/ml although the c.v. at 0.13 ng/ml of 21.9 % was higher than that recommended by Hunter, (1978). This imprecision was not considered clinically significant since the accurate estimation of elevated cTSH concentrations is of much greater importance than the measurement of low or normal values. Thus, in the clinically relevant region of the standard curve the precision was adequate. The LoD of 0.01 ng/ml is not considered to be sufficiently low to identify cases of hyperthyroidism or central hypothyroidism and is similar or identical to previous results (Meij *et al.*, 1996; Williams *et al.*, 1996; Peterson *et al.*, 1997) using the same kit. The identification of individuals with subnormal cTSH results is therefore not currently possible using this kit. The assay specificity was confirmed by demonstration of parallelism under dilution. The results varied from 88 to 108 % of the anticipated values which is similar to the findings of Williams *et al.*, (1996). The absence of a significant rise in cTSH after administration of GnRH also confirmed the specificity of the antibody used in the assay. The cTSH recovery concentrations from spiked samples reported by the manufacturer were consistent with good assay accuracy.

The validation of the TgAb assay was less detailed than for the other techniques in this chapter since the quantitative classification of results as either positive, negative or equivocal is not immediately suited to assessment of characteristics such as LoD or

dilutional parallelism. Since a numerical value is not assigned to the TgAb result it is impossible to assign a quantitative LoD. A value relative to the negative controls could be determined for these purposes but the value is of little clinical significance given that only abnormally increased TgAb results are of any importance. The intra-assay precision results were excellent when considered either based on the qualitative classification of samples or on the c.v.'s of their o.d.'s. However there was discordancy between the quantitative and qualitative results obtained during evaluation of inter-assay precision. The o.d. c.v.'s of each sample varied from 17 to 31 % and therefore were all inadequate according to standard validation criteria (Hunter, 1978). However the precision of actual result classification (i.e. either positive, negative or equivocal) was good to excellent. This study has demonstrated that the use of relatively broad qualitative categories, in addition to the safety margin of the equivocal category provides for good consistency of results despite considerable imprecision of o.d.'s within individual categories.

CHAPTER 4

ACHIEVING A DIAGNOSIS OF HYPOTHYROIDISM

4.1 LITERATURE REVIEW

The Healthy Thyroid Gland

Anatomical and Histological features

In the dog, the thyroid gland is a vascular bilobed structure located lateral to the proximal tracheal rings. It weighs 0.04 to 0.4 g/kg body weight and is located deep to the sternocephalicus muscle and therefore is not normally palpable. As in humans, accessory or ectopic thyroid tissue is common in the dog. Ectopic thyroid is present near the hyoid bone, along the cervical trachea or at the aortic base in over 50 % of dogs (Peterson & Ferguson, 1989; Capen, 1996). The thyroid gland is enclosed within a fibro-elastic capsule from which septae penetrate the gland dividing it into variously sized lobules. Blood and lymphatic vessels run within this connective tissue network (Wheater & Burkitt, 1987; Peterson & Ferguson, 1989). The lobules are composed of numerous follicles of varying size. Each follicle consists of a follicular lumen filled with colloid which is surrounded by a monolayer of cuboidal epithelial cells bounded by a fenestrated basement membrane. Colloid is a viscous gel containing thyroglobulin, a large molecular weight glycoprotein which is the precursor of the iodothyronines. The apical surface of the follicular epithelial layer has a microvillus structure similar to that of the intestinal brush border and projects into the follicular lumen. The outer surface contacts the surrounding blood vessels permitting the transfer of iodide and thyroid hormones between the follicular cells and the blood. In the mammalian thyroid gland a second endocrine cell type, C-cells, are found immediately below the basement membrane, between the follicular cells and between the follicles. These are responsible for the secretion of the polypeptide hormone calcitonin, involved in the maintenance of circulating calcium concentrations. C-cells are functionally distinct from the thyroid follicular units.

Synthesis of Thyroid Hormones

The only function of ingested iodine is the synthesis of iodothyronines within the thyroid gland (Peterson & Ferguson, 1989). This iodine is converted to iodide within the gastrointestinal tract before being absorbed into the circulation. A variety of processes, collectively known as the iodide transport mechanism are responsible for iodide entry into

the thyroid follicular cells. This is the first and rate limiting step in iodothyronine synthesis and is controlled primarily by the stimulatory effects of TSH (Ferguson, 1984; Vassart & Dumont, 1992; Taurog, 1996). The iodide transport mechanism is an active process and results in iodide accumulation within the thyroid follicular cytosol in concentrations approximately 10 to 200 fold greater than that found in the serum (Peterson & Ferguson, 1989). Intra-cellular iodide is oxidised to iodine and diffuses through the microvilli into the follicular lumen. Here it is incorporated within the tyrosine residues of thyroglobulin to form monoiodotyrosine (MIT) and diiodotyrosine (DIT) in a process known as organification (Werner, 1982). T_3 is produced by the coupling of one MIT with one DIT molecule and T_4 is produced by the coupling of two DIT molecules (Ferguson, 1984). The secretion of thyroid hormones begins with the fusion of colloid droplets containing thyroglobulin to the follicular cells. These are released into the follicular cytosol by pinocytosis and endocytosis (Greenspan & Rapoport, 1991; Dunn, 1996). Proteolysis of the droplets by lysosomal enzymes then liberates the iodotyrosines MIT and DIT and the iodothyronines T_3 and T_4. Deiodination of T_4 to T_3 occurs within the thyroid follicular cytosol and therefore although the stored ratio of T_4 to T_3 in the thyroid is approximately 12:1, the ratio entering the circulation is nearer 4:1 (Panciera, 1990a).

A variety of iodinated substances are released from the thyroid into the circulation including iodothyronines, iodotyrosines, iodoproteins and inorganic iodide. However, only the iodothyronines T_3 and T_4 are considered to have any significant biological activity. In healthy humans T_4 is produced in two to three fold greater quantities, and is cleared from the circulation at $1/20^{th}$ the rate of T_3 (Chopra, 1996). T_3 is therefore present in the serum at a much lower concentration than T_4. In contrast to T_4 which is derived entirely from thyroidal synthesis and secretion, only 20 % (humans) and 40-60 % (dogs) of circulating T_3 normally originates from direct thyroidal production. The remainder is produced by outer-ring deiodination of T_4 to T_3 by 5'-deiodinase (5'-D) in peripheral tissues especially the liver and kidneys (Ferguson, 1994; Kaptein *et al.*, 1994). This enzyme is also responsible for the conversion of rT_3 to 3,3'-diiodothyronine (3,3'-T_2). Peripheral deiodination of T_4 by 5-deiodinase (5-D) results in cleavage of an inner ring of the T_4 molecule with the production of rT_3. Because of its largely peripheral synthesis only 20 % of total body T_3 is present within the circulation, the remainder being located intracellularly (Kaptein *et al.*, 1994). T_3 is three to five times more metabolically active than T_4 leading some to suggest that T_4 acts essentially as a pro-hormone.

Two 5'-D isoenzymes exist. Type I is found in most tissues particularly liver, kidneys, muscle and skin and is responsible for peripheral conversion of T_4 to T_3 as described above. The type II 5'-D enzyme is located mainly in the brain, pituitary gland and brown smooth muscle and is responsible for the intra-pituitary deiodination of T_4 to T_3.

Control of Thyroid Hormone Synthesis and Secretion

Serum thyroid hormone concentrations are principally regulated by a negative feedback mechanism. The main stimulus for their synthesis and secretion is a rise in serum TSH concentrations which acts by binding to thyroid gland follicular cell surface receptors and stimulating cyclic adenosine monophosphate (cAMP) production (Ferguson, 1984; Vassart & Dumont, 1992; Rapoport & Spaulding, 1996). The binding of TSH to membrane receptors stimulates numerous intracellular pathways within the thyrocyte driving thyroid hormone synthesis and release (Rapoport & Spaulding, 1996).

TSH is synthesised and secreted from the pars distalis of the pituitary gland under the tonic simulation of TRH. The secretion of TSH from the pituitary is mainly inhibited by the negative feedback effect of the thyroid hormones, but a complicated network of neuropeptides and neurotransmitters including dopamine and somatostatin also modulate TSH release (Scanlon & Toft, 1996). The main inhibitor of TSH release is circulating free T_4 which enters the pituitary and is deiodinated to T_3 by the type II 5'-D isoenzyme. This T_3 acts via pituitary nuclear receptors to inhibit TSH synthesis and secretion by stimulating the production of an inhibitory protein and a decrease in the number of pituitary TRH receptors (Hinkle, Perrone & Schonbrunn, 1981). Thyroid hormones also inhibit the release of TRH by modifying TRH gene expression. This provides an additional mechanism for the regulation of TSH and thus thyroid hormone concentrations (Scanlon & Toft, 1996). Intrathyroidal mechanisms including the Wolff-Chaikoff blocking effect, altered thyroidal sensitivity to TSH and the relative production of T_3 to T_4 also modulate thyroid hormone synthesis, release and activity, but these are generally only of much relevance during periods of abnormal thyroid function. Certainly under normal conditions they are of much less importance in the regulation of thyroid function compared to the profound influence of circulating TSH concentrations (Scanlon & Toft, 1996; Feldman & Nelson, 1996).

Transport of Thyroid Hormones

The iodothyronines are present in the circulation mainly bound to the plasma proteins thyroid hormone binding globulin (TBG), albumin and thyroid hormone binding prealbumin

(TBPA (transthyretin)). A minor fraction of T_3 and T_4 is also carried by circulating lipoproteins (Robbins, 1996). In the healthy dog, approximately 60 % of T_4 is bound to TBG, 17 % to TBPA, 12 % to albumin and 11 % to the high density lipoprotein (HDL_2) (Larsson, Pettersson & Carlstrom, 1985). T_3 circulates bound to the proteins in a similar fashion. TBG becomes saturated once T_4 approaches 150 nmol/L and the other carrier proteins are virtually unsaturable (Larsson *et al.*, 1985). The binding affinity for thyroid hormones is considerably lower in dogs than in humans and consequently the free hormone fraction in dogs is greater than in humans. It is estimated that in canine serum the free or unbound fraction of T_4 is only 0.1-0.3 % of the total hormone concentration whilst approximately 1 % of total T_3 circulates unbound (Furth, Becker, Nunez & Reid, 1968; Refetoff, Robin & Fang, 1970; Feldman & Nelson, 1996). In contrast, the free T_4 and free T_3 fractions in humans are only approximately 0.03 % and 0.3 %, respectively. The reduced affinity of the thyroid hormones for their carrier proteins in dogs is responsible for the considerably more rapid hormone turnover in this species compared with humans. Correspondingly, the half life of T_4 is between 10 and 16 hours in dogs compared with about seven days in humans whilst the half life of T_3 in dogs is estimated to be only approximately five to six hours (Ferguson, 1994). The various thyroid hormone binding proteins vary in their concentration, affinity for thyroid hormones and dissociation rate constants. For example whilst TBG is the least abundant of the three major carrier proteins in human serum, it accounts for binding of approximately 70 % of the T_3 and T_4 compared with only 15-20 % to albumin, and 10-15 % to TBPA both of which are present in greater concentrations. In dogs the concentration of serum proteins is considerably lower than the corresponding human values. TBG concentration is particularly different between the species, with canine values approximating only 15 % of the human concentration. The concentration of total thyroid hormone in the dog is correspondingly reduced compared with humans (Refetoff *et al.*, 1970; Ferguson, 1994).

Actions of Thyroid Hormones

Thyroid hormones affect most body tissues by interaction with specific nuclear receptors although several non-nuclear pathways also exist (Oppenheimer, Schwartz & Strait, 1996). The thyroid hormone receptors act largely as transcription factors functioning in association with a broad range of other nuclear proteins to modify the expression of a diverse set of genes. At a cellular level, thyroid hormones therefore influence multiple metabolic processes from regulation of mitochondrial oxygen demand to the control of

protein synthesis. It is not surprising therefore that whilst some actions of thyroid hormones can be demonstrated within minutes to hours of thyroid hormone administration others may take weeks or even months to become apparent. The wide ranging biological role of thyroid hormones is partly responsible for the variable time required for the development of the different clinical signs associated with hypothyroidism and their resolution following thyroid hormone replacement therapy.

According to the free hormone hypothesis initially proposed by Robbins & Rall, (1957) it is only the non-protein bound hormone that is available for entry into cells with the protein bound hormone acting as a passive reservoir of hormone, serving to distribute it throughout the circulation. This concept is supported by studies of humans and animals with grossly disturbed (increased or reduced) total hormone concentrations resulting from genetic abnormalities in their thyroid hormone binding proteins. Such individuals may have grossly disturbed total hormone concentrations but relatively unaffected free hormone concentrations and do not develop clinical abnormalities consistent with thyroid disease, suggesting that the free hormone is ultimately responsible for thyroid status (Robbins, 1996). However, Pardridge, (1981) demonstrated single pass organ uptake of hormone in excess of the free hormone concentrations as determined by equilibrium dialysis. This was suggested to indicate protein-mediated tissue-specific hormone uptake. Despite this, the steady state uptake remained proportional to, albeit greater than, the free hormone concentration.

The Failing Thyroid Gland

Causes of Hypothyroidism

Primary Hypothyroidism

The most common adult form of human and canine hypothyroidism is primary or thyroidal hypothyroidism (Braverman & Utiger, 1996; Feldman & Nelson, 1996; Rijnberk, 1996). Worldwide, the most common cause in humans is chronic dietary iodine deficiency whilst in the developed western world it is usually the result of autoimmune thyroid disease (AITD), specifically chronic immune-mediated thyroiditis (Braverman & Utiger, 1996). The use of commercial dietary preparations has made iodine deficiency a rare occurrence in the dog. Approximately half of all canine cases result from lymphocytic thyroiditis whilst most of the remainder are caused by idiopathic thyroidal atrophy (Panciera, 1990a; Ferguson, 1994; Kemppainen & Clark, 1994). Primary thyroidal failure occasionally results from neoplastic

destruction, anti-thyroid medication, radiation therapy, or congenital defects although these are rare in dogs (Kemppainen & Clark, 1994; Braverman & Utiger, 1996; Rijnberk, 1996). Primary hypothyroidism is associated with subnormal thyroid hormone concentrations. The negative feedback which these thyroid hormones normally exert on pituitary TSH synthesis and secretion is therefore reduced and primary hypothyroidism is generally associated with elevated circulating TSH concentrations.

Histologically, lymphocytic thyroiditis is characterised by multifocal or diffuse infiltration of the thyroid gland by lymphocytes, macrophages and plasma cells. The follicular epithelial cells which are normally cuboidal in appearance become columnar under the stimulatory effect of increased TSH concentrations. There is thickening of the basement membrane and replacement of normal thyroid tissue with mature fibrous connective tissue (Gosselin, Capen & Martin, 1981a; Lucke, Gaskell & Wotton, 1983; Kemppainen & Clark, 1994). This process is progressive and it has been suggested that total thyroidal destruction may occur over a three to four year period after which time there remains virtually no functional thyroid tissue (Belshaw, 1983). It is not until approximately 75 % of the gland is destroyed that functional failure develops and clinical signs of hypothyroidism become apparent (Peterson & Ferguson, 1989).

Whilst the aetiology of canine lymphocytic thyroiditis is believed to be immune mediated, analogous to the AITD syndrome in humans, the underlying cause is unclear. Thyroiditis has been reported to be more prevalent in certain breeds and family lines of dogs (Conaway, Padgett & Nachreiner, 1985) and this subject is discussed in detail in Chapter 7. Biochemical evidence of thyroiditis is also more common in apparently healthy dogs closely related to individuals with evidence of thyroiditis as determined by TgAb estimation (Haines, Lording & Penhale, 1984b). It is therefore presumed that hypothyroidism resulting from thyroiditis has a genetic component but other predisposing factors remain to be identified. Not surprisingly therefore, a breed predisposition to hypothyroidism has also been reported in the literature although the populations under test and the diagnostic criteria for the confirmation of hypothyroidism have varied between reports making this issue less clear. This is discussed in detail in Chapter 5.

Idiopathic thyroidal atrophy is characterised by reduction in follicular size and replacement of the normal parenchymal tissue with adipose connective tissue. There is degeneration of follicular cells with exfoliation into the colloid and interfollicular area. Like lymphocytic thyroiditis the underlying cause of this process is unknown but histologically it appears to be a non-inflammatory degenerative process (Gosselin, *et al.*, 1981a). The

degeneration has been suggested to be the end stage of an autoimmune process (Rijnberk, 1996). However, histopathological examination of affected glands rarely demonstrates a concurrent inflammatory infiltrate which has been suggested to indicate that the aetiology differs from that of lymphocytic thyroiditis (Gosselin *et al.,* 1981a).

Central Hypothyroidism

Spontaneous secondary or central hypothyroidism reputedly accounts for under 5 % of adult cases and is caused by a failure of normal TSH secretion by the thyrotropic cells of the pituitary gland (Feldman & Nelson, 1996). The absence of thyroidal stimulation results in atrophic degeneration of the thyroid characterised by follicular distention and flattening of the follicular epithelium. This can be distinguished from the changes typical of the more common primary idiopathic thyroidal atrophy (Rijnberk, 1996). The most common spontaneous cause of central hypothyroidism in the dog is believed to be a tumour within or adjacent to the pituitary (Rijnberk, 1996). However there are few reports of this type of hypothyroidism in adult dogs (Chastain, Riedesel & Graham, 1979). More commonly central hypothyroidism results from suppression of pituitary TSH secretion by exogenous glucocorticoid administration or spontaneous hyperadrenocorticism (Peterson *et al.,* 1984). However, this is usually a temporary and reversible condition in which treatment of the underlying cause is curative and thyroid hormone supplementation is not required (Peterson *et al.,* 1984).

Diagnosing Hypothyroidism

The diagnosis of hypothyroidism in humans is relatively straightforward with the measurement of serum free T_4 and endogenous TSH concentrations providing a highly sensitive and specific assessment of thyroid status (Stockigt, 1996). By contrast the diagnosis of this disease in the dog is controversial and is frequently the cause of considerable confusion. A variety of tests to confirm canine hypothyroidism have been employed, most of which have their origins in human medicine and this in itself has sometimes been responsible for their poor performance. In addition, various technical problems inherent in some of the commonly used assays, and the variation in diagnostic classification criteria employed between reports has perplexed both practitioners and researchers alike.

Clinical and Routine Clinicopathological Features

There are various well recognised laboratory abnormalities associated with hypothyroidism although these abnormalities are non-specific findings and their presence or absence cannot confirm or exclude a diagnosis of hypothyroidism. Similarly, the historical and clinical abnormalities are both variable and non-specific for the disease. This subject is discussed in detail in Chapter 5.

Thyroid Hormone Concentrations

This review will briefly summarise the major tests used to diagnose hypothyroidism, and discuss in more detail those reports where the performance of these tests is compared.

Total Thyroxine

The principal product of the thyroid gland is T_4 and basal serum total T_4 estimation has traditionally been the mainstay of the diagnosis of canine hypothyroidism (Panciera, 1990b). As described in Chapter 3, there is good cross reactivity of T_4 between most species making the commercially available human total T_4 assays suitable for use with canine samples once modified to allow measurement of the lower circulating concentrations in the dog (Belshaw & Rijnberk, 1979; Li *et al.*, 1986; Panciera *et al.*, 1989).

There is universal agreement that circulating total T_4 is usually subnormal in hypothyroidism. Belshaw & Rijnberk, (1979) confirmed hypothyroidism in 48 dogs using either a modified TSH stimulation test based on ^{131}I uptake before and after TSH administration, or gamma camera visualisation of the thyroid glands 30 minutes after pertechnetate administration. Total T_4 was measured as described previously. Reference limits were defined as the 2.5^{th} and 97.5^{th} percentiles of results from 126 healthy dogs and results were considered abnormal if they were outwith the central 90^{th} percentile of this reference distribution. All hypothyroid dogs had subnormal total T_4 results.

Nelson *et al.*, (1991) reported resting circulating total T_4 concentrations that were below the laboratory reference limits in 50 of 51 (98 %) hypothyroid dogs categorised by TSH response testing (total T_4 measurement immediately before and six hours after the intravenous administration of 0.1 i.u./kg body weight exogenous TSH). The method used for total T_4 estimation was a human RIA kit (Clinical Assays) previously validated for use with canine serum by Evinger, Nelson & Bottoms, (1985). One hypothyroid dog had an inappropriate total T_4 value of 39.9 nmol/L. The authors attributed this result to the presence of T_4Ab and confirmed lymphocytic thyroiditis in that individual.

A number of reports have simultaneously evaluated multiple tests of thyroid function including total T_4 and these are summarised below.

Peterson *et al.*, (1997) and Scott-Moncrieff *et al.*, (1998) both used the same TSH response test protocol to confirm hypothyroidism (circulating total T_4 measurement immediately before and six hours after administration of 0.1 i.u. TSH/kg body weight). Peterson *et al.*, (1997) reported depressed circulating total T_4 in 48 of 54 (89 %) hypothyroid dogs, and Scott-Moncrieff *et al.*, (1998) reported subnormal basal total T_4 concentrations in all of 16 hypothyroid dogs. Both studies measured total T_4 using a coated tube RIA (Coat-A-Count Canine T_4, Diagnostic Products). Peterson *et al.*, (1997) generated a reference range for total T_4 by selecting the 5^{th} to 95^{th} percentiles of all results from 150 healthy dogs. Results outwith this range were considered abnormal. Scott-Moncrieff *et al.*, (1998) used a laboratory reference range to interpret the test results. Whilst six of the hypothyroid dogs reported by Peterson *et al.*, (1997) had total T_4 values within or above the reference range, three of these cases had spuriously elevated results caused by T_4Ab whilst in the remaining three cases concentrations were in the low-normal range. Thyroid hormone autoantibodies cause spurious results with most thyroid hormone immunoassays. The type of interference depends on the method of separation of bound from unbound hormone employed in the assay system but in most commercial kits this usually causes artefactually elevated results. The presence of antibodies to the hormone in a competitive assay system allows binding of the tracer to the endogenous Ab. This tracer-Ab complex cannot compete with endogenous hormone for assay binding sites and following removal of unbound hormone and the tracer-Ab complexes there is an artefactual increase in the measured hormone concentration.

Whilst the diagnostic sensitivity of total T_4 estimation for hypothyroidism is undoubtedly high, the specificity of this analyte is particularly poor for a variety of reasons. First, normal random fluctuation in total T_4 concentrations occurs in healthy dogs and subnormal values are a common occurrence during health. Kemppainen & Sartin, (1984) measured serum total T_4 at 20 minute intervals from 11 euthyroid dogs using an unspecified RIA (Diagnostic Products). There was episodic fluctuation of secretion in all dogs which had no predictable pattern. No indication of the number of dogs with, or the definition of, "subnormal" results, was provided by the authors. More recently, Miller, Nelson, Scott-Moncrieff, Neal & Bottoms, (1992) performed serial total T_4 estimation using an unspecified RIA (Clinical Assays) over a 12 hour period in 20 clinically healthy euthyroid dogs and reported subnormal (less than the laboratory reference value, i.e. 20

nmol/L) total T_4 concentrations in 10 of the animals on at least one occasion. Second, there is a progressive decline in mean serum total T_4 concentrations with increasing age such that juvenile dogs less than three months of age have total T_4 concentrations approximately two to five times that of adults. Reimers *et al.,* (1990) documented a statistically significant decrease in total T_4 values (unspecified RIA, ICN Biomedicals/Micromedic Systems) with age in 1048 dogs categorised as one of six age groupings between one week and 11 years. Mean (± s.e.m.) total T_4 concentrations were 39.1 ± 0.6 nmol/L (one to six weeks of age), 25.0 ± 0.6 nmol/L (six to 12 weeks), 25.1 ± 0.5 nmol/L (three to six months), 24.4 ± 0.8 nmol/L (six to 12 months), 22.1 ± 0.6 nmol/L (one to six years) and 19.3 ± 0.6 nmol/L (six to 11 years) in the various groups. Third, the same study also evaluated the effect of breed size on serum total T_4 concentrations and identified lower values in medium (25.0 ± 0.5 nmol/L) and large-breed (26.1 ± 0.4 nmol/L) dogs compared with small breed (31.5 ± 0.8 nmol/L) dogs. Gaughan, Bruyette & Jordan, (1996) also demonstrated an effect of breed on total T_4 concentration (unspecified method) with greyhounds having significantly depressed values compared with non-greyhound pet dogs.

The influence of gender on thyroid hormone concentrations has been evaluated in several studies with conflicting results. Le Roux, (1983) demonstrated that mean (± s.d.) total T_4 concentrations (30.6 ± 5.6 nmol/L) were significantly lower in female dogs 55 weeks following ovariectomy compared to those which had received a sham ovariectomy (39.5 ± 12.1 nmol/L). A similar effect has also been reported in male dogs following castration (Ganong & Junker, 1955). Gosselin, Capen, Martin & Targowski, (1980) reported no effect of hyperoestrogenism on total T_3 or T_4 concentrations (unspecified RIA) in six dogs. Reimers *et al.,* (1984) evaluated circulating total T_4 and total T_3 concentrations using coated-tube RIA's (Autopak, Micromedic systems) in five anoestrus, five pro-oestrous, five dioestrous, five pregnant and five lactating beagle bitches and five male dogs. Total T_4 concentrations were significantly increased in dioestrous and pregnant bitches and total T_3 was significantly increased in dioestrous bitches compared with each other group of dogs. Unfortunately, summary mean values were not provided for each group, but broadly speaking, mean total T_4 concentrations were approximately 30 nmol/L and 25 nmol/L during dioestrus and pregnancy, respectively, compared with approximately 15 nmol/L during most other stages of the oestrus cycle. Similarly, mean total T_3 concentrations were approximately 1.5 nmol/L during dioestrus compared with approximately 0.92 nmol/L during most other stages of the oestrus cycle. There were no significant differences between other groups suggesting that progesterone dominated

phases of the oestrous cycle are associated with increased thyroid hormone concentrations. The effect of gender on thyroid hormones was also evaluated by Kemppainen & Sartin, (1984) who reported significantly lower mean (± s.d.) total T_4 concentrations in intact male (15.4 ± 3.6 nmol/L) compared with anoestrus intact female (20.7 ± 3.6 nmol/L) dogs. Whilst the magnitude of variation in thyroid hormone concentrations between reproductive groups is greater in the report by Reimers *et al.,* (1984) than in the study by Kemppainen & Sartin, (1984), both studies have groups with mean results of approximately 15 nmol/L. Using most conventional criteria these results are bordering on "subnormal" and therefore reproductive factors apparently have the potential to be of clinical relevance. Reimers *et al.,* (1990) reported no significant difference in mean (± s.e.m.) circulating total T_4 concentrations in 517 male dogs (26.8 ± 0.5 nmol/L) compared with 557 females (27.2 ± 0.5 nmol/L) although no account was taken of the stage of the reproductive cycle in the female animals. The reason for the difference in results between some of these studies is unclear.

Based on the above studies evaluating the effects of random fluctuation, age, breed and gender on circulating thyroid hormone concentrations it is clear that normal physiological mechanisms in the healthy dog exist which cause considerable variation in total T_4 concentrations.

The overlap which exists between hypothyroid dogs and dogs with NTI and those receiving certain drug therapies is even greater than the overlap with healthy dogs. This subject is discussed in detail in Chapter 8. This further reduces the diagnostic specificity of total T_4 measurement for hypothyroidism.

Given the various factors other than hypothyroidism capable of decreasing total T_4 concentrations, estimation of this analyte is only truly diagnostic if concentrations are within the reference range in which case hypothyroidism can generally be excluded.

Total Triiodothyronine

Serum total T_3 estimation is infrequently performed in the evaluation of hypothyroidism since the overlap of results from euthyroid dogs with NTI and hypothyroid dogs tends to be even greater than that reported for total T_4 (Panciera, 1994). Using the diagnostic criteria outlined above, Belshaw & Rijnberk, (1979) reported plasma total T_3 to be of about equal value to that of total T_4 in discriminating between 126 healthy and 48 hypothyroid dogs. Thyroid hormones were measured using charcoal separation RIA methods with unspecified commercial T_4 and T_3 antisera (Nichols institute) and labelled hormones

(Sigma). However, measurement of total T_3 from dogs with clinical disease reduces its diagnostic specificity for hypothyroidism.

Nelson *et al.*, (1991) reported that mean (± s.d.) total T_3 was not significantly different between 51 hypothyroid (0.77 ± 0.77 nmol/L), 62 healthy (1.08 ± 0.46 nmol/L) and 59 euthyroid dogs with one of eight categories of NTI. The method used for total T_3 estimation was a human RIA kit (Clinical Assays) previously validated for use with canine serum by Evinger *et al.*, (1985). Cases were categorised using a combination of clinical signs, TSH response tests, thyroid gland histopathological examination, response to therapy and case follow up. Mean thyroid hormone results from the NTI cases were categorised into each of the eight types of diseases but an overall mean value was not provided for these dogs. Nevertheless, total T_3 concentrations ranged from 0.15 to 3.07 nmol/L in the dogs with NTI, compared with 0.15 to 1.84 nmol/L (healthy dogs) and 0.15 to 2.76 nmol/L (hypothyroid dogs).

Miller *et al.*, (1992) determined total T_3 concentration using the same method as Nelson *et al.*, (1991) in 19 hypothyroid, 20 healthy and 18 euthyroid dogs with atopic dermatitis, categorised by TSH response tests and reported no significant difference in results between the three groups. Overall mean total T_3 concentrations for each group were not provided, since the study was designed to evaluate the effect of time of sampling on hormone concentration, and consequently reported the mean values at each individual time point during the study. Random fluctuation of total T_3 was common with 14 of 20 healthy euthyroid, and 15 of 18 sick euthyroid dogs (with atopic dermatitis) having a subnormal concentration (less than the laboratory reference value, i.e. < 1.0 nmol/L) on at least one occasion over a 12 hour sampling period.

Recently, Peterson *et al.*, (1997) reported a median total T_3 concentration of 1.4 nmol/L in 31 hypothyroid dogs which was not significantly different to 1.5 nmol/L in 135 healthy or 1.2 nmol/L in 38 euthyroid dogs with NTI. Total T_3 was measured using a coated tube RIA (Coat-A-Count Canine T_3, Diagnostic Products) and cases were categorised based on the results of TSH response tests. In that study, subnormal total T_3 results occurred in only three hypothyroid dogs although five of the 31 cases had elevated results due to autoantibody interference.

Several factors are presumed to be responsible for the poor diagnostic performance of total T_3 estimation in hypothyroidism. First, the thyroid gland itself is responsible for the secretion of only approximately half of circulating T_3. Consequently, the measurement of circulating total T_3 is a relatively insensitive method with which to evaluate changes in

thyroid gland function. Second, most T_3 is produced peripherally within the tissues by 5' deiodination of T_4 Consequently, any changes in the rate of peripheral T_3 production, such as occurs in NTI, will alter circulating total T_3 concentration but not necessarily reflect thyroid gland function. This may contribute to the poor correlation of circulating total T_3 concentration and thyroid gland function. Third, the failing thyroid gland is associated with an increase in endogenous TSH concentration. This stimulates the preferential thyroidal secretion of T_3 instead of T_4 (Peterson & Ferguson, 1989; Panciera, 1990a). This presumably helps maintain circulating total T_3 concentrations and therefore metabolic activity in the face of impending failure (Lum, Nicoloff, Spencer & Kaptein, 1984). However, as a consequence of this, decreases in circulating T_3 values, particularly in the early stages of thyroid dysfunction, are inconsistent.

Free Thyroid Hormones

The measurement of free thyroid hormone concentrations should in theory at least, offer considerable advantages over total hormone estimations. Assuming the free hormone hypothesis to be broadly correct, free hormone is the metabolically active fraction available for tissue uptake and therefore should provide the most accurate assessment of cellular thyroid status (Robbins & Rall, 1957). Additionally, measurement of free hormone concentration is theoretically unaffected by the presence of thyroid hormone autoantibodies, the presence of protein binding inhibitors, or alteration in the concentration and/or affinity of thyroid hormone binding proteins, all of which interfere with total T_4 estimation. The close correlation between free T_4 concentrations and metabolic status has resulted in its measurement being used as the gold standard test of thyroid function in humans (Ekins, 1985).

An indirect assessment of free T_4 concentration, the free thyroxine index (fT_4I) has been widely used in humans, and more briefly in dogs (Kallfelz, 1968; Kallfelz & Erali, 1973). The fT_4I is the product of the thyroid hormone binding ratio (THBR) and circulating total T_4 concentration. The THBR, previously known as the "T_3 resin sponge uptake test" is based on the in-vitro competition between plasma proteins and an ion exchange or a charcoal resin matrix for endogenous serum thyroid hormones and added T_3 tracer. Radiolabelled T_3 is used in preference to T_4 because it has a lower affinity for human TBG and therefore equilibrates more rapidly in vitro. The degree of tracer binding to the resin is inversely proportional to the concentration of unoccupied protein binding sites and their affinity for thyroid hormones. In humans the THBR often correlates reasonably accurately

with free T_4 concentrations as measured by dialysis (Ferguson, 1994). Unfortunately the fT_4I is very insensitive to changes in thyroid status in dogs largely due to the much lower concentration and affinity of circulating TBG for thyroid hormones in dogs compared to humans. Consequently the use of the THBR and fT_4I have been discarded in the dog.

Currently the measurement of so-called free T_4 is usually performed by either dialysis, ultrafiltration, or free hormone RIA methods. Direct immunoassay has been by far the most commonly used method. However many of these techniques have been severely criticised on theoretical and practical grounds and reports of their validation and clinical performance have caused both confusion and controversy (Ekins, 1983; Alexander, 1986). A comprehensive review of the practical and physicochemical properties of the available methodologies has been previously reported by Ekins, (1987; 1993).

Three "free hormone" RIA methods have been utilised for the measurement of thyroid hormones in humans, namely the "labelled hormone antibody uptake", "labelled hormone back titration" and "labelled analogue" techniques.

The labelled hormone antibody uptake method relies upon estimation of total sample hormone concentration followed by the estimation of the fraction of the hormone which binds to exogenous antibody. This is in essence similar to the traditional fT_4I estimates. The principal disadvantage of this technique is that the amount of hormone being removed from the assay system and incorporated in the antibody-ligand complex is relatively large. This results in progressive dissociation of previously bound hormone much as occurs during sample dilution discussed below. This feature can be responsible for erroneous values in situations such as the presence of thyroid hormone protein binding inhibitors (THBI).

The labelled hormone back titration method is a two step procedure. First solid-phase antibody is incubated with the sample allowing the ligand to bind. The antibody is then removed and washed, and then incubated in the presence of labelled hormone. The labelled hormone attaches to any unoccupied antibody binding sites allowing inference of the free hormone binding and therefore concentration. This method is not subject to the problems encountered with the single step "analogue" assays described below, and the validity of this method is not questioned. However, this methodology has been subject to unacceptable intra-assay drift arising from the variable sequential incubation time from one sample to the next. This effect can be minimised by the selection of an antibody with appropriate reaction kinetics but nevertheless has proved to be a problem in practice.

By far the most widely used method over the previous 20 years has been the labelled analogue RIA's in which a labelled T_4 analogue competes with endogenous free T_4 for a limited number of solid-phase Ab binding sites (Montgomery, Nelson, Ferguson & Feldman, 1991; Nelson *et al.*, 1991; Paradis, Page, Lariviere & Fontaine, 1996). Whilst the theoretical basis for the technique is perfectly sound, the assays rely upon the absence of any interaction between the labelled T_4 analogue and endogenous serum proteins. However it is now clear that in practice this fundamental requirement is not met (Stockigt, DeGaris, Csicsmann, Barlow, White & Hurley, 1981; Bayer, 1983; Ekins, 1985). The main effect of binding of the analogue to serum proteins is the potential to change the concentration of analogue which is available for antibody binding. Clearly the greater the binding of labelled analogue to endogenous serum proteins, the lower the concentration of analogue available for binding to the anti-T_4 antibody. Similarly any reduction in the concentration of binding proteins or their affinity for the analogue, increases the free analogue concentration and consequently competition between analogue and endogenous hormone. This causes a decrease in the measured free hormone concentration.

The presence of THBI in NTI causes displacement of hormone from binding proteins and therefore, free T_4 concentration increases. This has been confirmed using equilibrium dialysis techniques (Chopra, Solomon, Hepner & Morgenstein, 1979a). This is typically a short-lived phenomenon since the absolute free T_4 concentration re-equilibrates fairly rapidly as TSH production and consequently total T_4 values decrease, returning free T_4 concentration to normal despite an increased free hormone fraction. However, when using analogue methods in which there is significant interaction between the analogue and endogenous proteins, the presence of THBI also displaces analogue from the protein, thereby increasing the competitive effect of the analogue for assay Ab. This artefactually reduces the measured free T_4 concentration compared to the "normal" results obtained with that assay. In clinical situations such as chronic NTI in which THBI are present (Chopra, Chua Teco, Nguyen & Solomon, 1979b), the measured free T_4 value depends largely on the interaction of analogue and serum proteins and frequently bears little relation to the true free hormone concentration. This is the principal reason why one step analogue assays are unreliable when used in cases with NTI. Whilst the increase in true free T_4 fraction and reduction of analogue-protein binding can be anticipated, the magnitude of the effects of THBI cannot be determined without knowledge of the relative interaction of both THBI and analogue for each of the binding proteins in the serum samples and the kit reagents. Since the latter is unknown, and almost certainly different between each of the analogue

assays, the kits' response to these competitors cannot be accurately predicted. Furthermore, species differences in normal binding proteins may potentially alter results depending on the relative affinity of the analogue for different proteins.

Various chemical blockers have been added to the analogue systems in an attempt to prevent binding of the labelled analogue with plasma proteins (Witherspoon, El Shami, Shuler, Neely, Sonnemaker, Gilbert & Alyea, 1988). However, these modifications have been found to be broadly cosmetic and have not been effected by identifying an analogue with the desired physicochemical characteristics, but by altering the other assay components to give broadly correct results in a number of specific clinical situations (especially pregnancy which is associated with markedly elevated TBG concentration in humans). Using one such assay (Amerlex FT_4 RIA, Amersham) Bayer, (1983) demonstrated that measured free T_4 results in hormone-free serum were in fact directly proportional to the concentration of albumin in the samples.

Further doubt has been cast on the validity of the analogue assays based on results of experimental dilutional studies. When serum is diluted with an aqueous inert diluent there is a net dissociation of bound hormone resulting in an approximate maintenance of the free hormone in the diluted medium. Dilution therefore causes a reduction in the total hormone concentration far in excess of the reduction in free hormone concentration. Near constancy of assay results in the face of serum dilution is now widely regarded as the classic test of free T_4 assay validity. Dilution of samples in this manner, results in depletion of the free hormone pool to an extent dependent on the normal proportion of bound and free hormone. In the case of T_4 specifically, dilutions in the order of 500 to 1000 result in imperceptible decreases in free T_4 measured by dialysis methods (Ekins, 1987). In the case of less highly protein bound hormones such as T_3 (approximately 1 % free) or steroids (up to 10 % free) the same dilution will have an effect that is orders of magnitude greater. A similar variation in free hormone depletion as a consequence of dilution may be expected when comparing results from humans and dogs, since the free T_4 fraction is approximately three fold greater in dogs. Nevertheless, the relative free T_4 fraction in dogs remains minimal and under normal circumstances, assuming no indirect dilution effects such as those discussed above, dilution is thus of little consequence in this species. The extent of dilution inherent within an assay system should therefore take account of the maximum "permissible" dilution factor, depending on the species and hormone being evaluated. This potential error arises in all free hormone methodologies, not simply analogue techniques.

However, the failure of analogue methods to pass this "dilution test" provides further evidence that they are not truly free hormone assays (Ekins, 1985).

The effect of dilution of samples containing THBI prior to free T_4 estimation using an analogue methodology is complicated. Dilution of the sample will alter the effects exerted by the THBI. In general, if a THBI interacts with protein binding sites with a lower affinity than does the endogenous hormone, dilution will tend to reduce the effects of the THBI. This will typically reduce the measured free T_4. However, if the THBI reacts with the binding sites with a higher affinity than the hormone, the converse applies (Ekins, 1985). Clearly the one-step analogue method remains fundamentally incorrect and liable to artefact (Ekins, 1985). Consequently whilst analogue assays tend to give results which correlate with free T_4 measured by equilibrium dialysis in healthy patients, they are consistently lower when used in cases with NTI and their use is now discouraged (Ekins, 1985; Montgomery *et al.,* 1991; Ferguson, 1994).

The reference techniques for free hormone measurement are equilibrium dialysis and ultrafiltration. However there remain practical difficulties associated with both of these methods. Ultrafiltration in particular is not suitable for routine clinical use and consequently is not widely commercially used for thyroid hormone estimations (Schussler & Plager, 1967). Ultrafiltration does have the advantage that it can be performed without sample dilution and is therefore not associated with some of the problems inherent in the conventional RIA and dialysis techniques. However, ultrafiltration is complicated by the presence of any non-filtrable THBI which if confined to the protein-containing filtrand will have the same effects described below.

Dialysis techniques rely on bringing the serum ("dialysand") into contact with a buffer ("dialysate"), the two being separated by a membrane which is permeable to free hormone but not to the hormone binding proteins. Free hormone passes through the membrane causing dissociation of protein-bound hormone to maintain the thermodynamic equilibrium within the system. Once a state of equilibrium is reached the concentration of free hormone on both sides of the membrane is identical. Measurement of free hormone in the dialysate by either direct or indirect methods can then be performed without the complicating effect of endogenous proteins. Indirect measurement of dialysate hormone concentration was first performed by Sterling & Hegedus, (1962). This method relies on estimation of the total hormone in the sample, for example by conventional RIA, followed by measurement of the fraction of hormone which appears in the dialysate. This is achieved by the addition of tracer to the original sample prior to dialysis, and calculation of the

percentage radioactivity which is ultimately dialysed. The principal limitation of this approach has been associated with impurities within the radioactive tracer. Such impurities enter the dialysate and contribute to a falsely elevated free T_4 estimation. This is of particular importance in the case of the thyroid hormones since the free hormone fraction is normally extremely small. The presence of even small quantities of non-T_4 tracer therefore causes a considerable relative overestimation of free T_4. As a consequence of the problems associated with tracer impurities, direct dialysate hormone measurement were subsequently developed. Ellis & Ekins, (1973) reported a direct dialysis method for free T_4 and free T_3 estimation. This was apparently designed using highly specific antisera and computer optimisation techniques to maximise the sensitivity of the assay. However, no further validation data were provided. Free T_4 results ranged from 32.2 to 51.5 pmol/L in healthy individuals. Subnormal results in hypothyroidism, and increased concentrations in thyrotoxicosis were reported but detailed data of results or categorisation methods were not provided. Helenius & Liewendahl, (1983) designed a method of sample dialysis followed by direct RIA of free hormone in the dialysate utilising disposable dialysis cells. These were manufactured from thermoplastic resin which had minimal absorptive capacity for T_4 yet, unlike glass, was sufficiently inexpensive to allow mass production of the cells in a disposable format. The dialysis cells containing 100 µl sample and 400 µl buffer were incubated for 18 hours at 37°C on a rack shaker. The dialysis membrane was manufactured from Visking tubing (molecular mass cut off 6000). Direct competitive RIA of the dialysate was then performed on 600 µl dialysate using commercial T_4 antibody (International Laboratory Services) and ^{125}I labelled T_4. Bound and unbound hormone were separated using PEG precipitation. The assay was compared with a number of non-dialysis commercial free T_4 methods, and generally gave results which were greater than obtained with the other methods, particularly in cases with NTI. Nelson & Weiss, (1985) developed a highly sensitive T_4 RIA to allow the direct determination of free T_4 in dialysates. The assay was designed to maintain the pH and concentration of ions known to affect T_4 binding to serum proteins by the combined use of HEPES buffer and HEPES acid (Calbiochem-Behring). Progressive serum dilution prior to dialysis resulted in a decrease in measured free hormone in patients with NTI but not in serum from healthy individuals. The magnitude of the decrease in the patients with various NTI varied widely and was not predictable. Nelson & Tomei, (1988) evaluated a similar dialysis method to that reported earlier by Helenius & Liewendahl, (1983) utilising the sensitive RIA described by Nelson & Weiss (1985). The working range of the assay was reported to be 2.6 to 164.7 pmol/L with

a reference range of 10.3 to 34.7 pmol/L. Free T_4 concentrations were reported to be 41.2 to 615.2 pmol/L in hyperthyroidism, 11.6 to 34.7 pmol/L in excess TBG and familial dysalbuminaemic hyperthyroxinaemia, less than 2.6 to 9.0 pmol/L in hypothyroidism, 11.6 to 32.2 pmol/L in severe TBG deficiency, and 10.3 to 45.0 pmol/L in various NTI. The cells designed for this purpose were modified and ultimately became commercially available in kit form (Free T_4 by Equilibrium Dialysis, Nichols Institute Diagnostics).

Ferguson & Peterson, (1992) evaluated a free T_4 method which was a modification of the indirect dialysis technique reported by Sterling & Brenner, (1966). Serum was collected from 42 dogs with hyperadrenocorticism and 103 healthy dogs. The mean (± s.e.m.) free T_4 concentration was significantly lower in the dogs with hyperadrenocorticism (16.9 ± 2.3 pmol/L) compared with the healthy dogs (23.6 ± 1.0 pmol/L). The free T_4 fraction was significantly higher in the dogs with hyperadrenocorticism (0.15 ± 0.018 %) compared with the healthy dogs (0.09 ± 0.003 %) indicative of a reduction in serum protein binding.

Panciera & Post, (1992) evaluated the effect of trimethoprim and sulphadiazine medication on free T_4 concentrations determined using a modified equilibrium dialysis kit method (Free T_4 by Equilibrium Dialysis, Nichols Institute Diagnostics) in 12 healthy adult beagle dogs. Whilst no effect of the drug at standard therapeutic doses was recognised, apparently random decreases in free T_4 concentration occasionally occurred. The actual free T_4 concentrations in the treated and control groups were difficult to determine due to the errors in the manuscript discussed in Chapter 3.

Scott-Moncrieff, Nelson, Ferguson & Neal, (1994) determined circulating free T_4 concentrations using the same kit method as Panciera & Post, (1992) in 31 healthy, 11 hypothyroid and 32 euthyroid dogs with NTI. Cases were categorised based on history, physical examination, TSH stimulation tests and the need for thyroid hormone replacement therapy. Mean (± s.d.) free T_4 concentration was significantly higher in the healthy (25.74 ± 10.3 pmol/L) dogs compared to the hypothyroid (2.57 ± 2.57 pmol/L) animals. The euthyroid group with NTI were categorised into those with hyperadrenocorticism (n=10), obesity (n=9), hypoalbuminaemia (n=8) and megaoesophagus (n=5). Only those dogs with hyperadrenocorticism had free T_4 concentrations significantly lower than the healthy animals. The accuracy of free T_4 for correctly identifying cases as either hypothyroid or euthyroid was 0.85.

Peterson *et al.,* (1997) evaluated the same free T_4 method in 54 dogs with hypothyroidism, 54 dogs with various NTI and 150 clinically healthy dogs. Cases were

categorised based on the results of bovine TSH response tests (total T_4 estimation prior to and six hours following the intravenous administration of 0.1 i.u. TSH/kg body weight). Median free T_4 concentrations were significantly lower in the hypothyroid dogs (2 pmol/L) compared to the euthyroid dogs with NTI (20 pmol/L) and the healthy dogs (22 pmol/L). Fifty three of the 54 (98 %) hypothyroid dogs had free T_4 concentrations less than the reference range (derived from the healthy dogs as indicated above) compared to only four of the 54 (7 %) euthyroid dogs. The diagnostic sensitivity, specificity and accuracy of free T_4 estimation for hypothyroidism was 0.98, 0.93 and 0.95, respectively.

Thyrotropin

In human medicine, the measurement of circulating TSH concentration generally provides an accurate indication of functional thyroid status due to the exquisite sensitivity of the pituitary thyrotrophs to changes in circulating free T_4 concentration. In fact when plotted together, TSH is usually depicted on a logarithmic scale because of its dramatic response to changes in serum free T_4 (Stockigt, 1996). The human TSH assays currently used are third generation assays and are both extremely sensitive and accurate. These assays are not only capable of distinguishing low, normal and high values, but can differentiate the low values found in NTI from the very low values associated with thyrotoxicosis (Spencer, LoPresti, Patel, Guttler Eigen, Shen, Gray & Nicoloff, 1990).

Chastain, (1978) first reported on the use of a human TSH assay as a diagnostic test for canine hypothyroidism using 14 clinically healthy and five suspected hypothyroid dogs. Four of the hypothyroid group had clinical signs consistent with hypothyroidism, and a subnormal total T_4 response to TSH administration. One other dog was categorised as hypothyroid based on a subnormal basal total T_4 concentration alone. The study found no correlation of cTSH results with categorisation, and concluded that the cross-reactivity of the cTSH molecule, most likely the β-subunit with the antibody used in the assay, was insufficient to be clinically useful. In a larger series of dogs Larsson, (1981) investigated a human based assay in which the cTSH Ab was derived from sheep. Bovine TSH response tests were used to classify cases, and confirmed hypothyroidism in 40 of 150 dogs investigated. The mean (± s.d.) cTSH concentration in the hypothyroid group (9.49 ± 6.73 μU/ml) was significantly higher than in a random selection of 20 of the euthyroid dogs (3.5 ± 1.67 μU/ml). However, there was marked overlap of results between euthyroid and hypothyroid dogs reducing the diagnostic utility of the assay. In the same study TRH response testing was performed in eight dogs and revealed minimal increase in cTSH

concentrations. This was interpreted as an indication of inadequate cross-reactivity of the Ab used in the assay. Quinlan & Michaelson, (1981) reported the use of a supposedly canine-specific cTSH assay based on rabbit derived cTSH Ab in dogs following propylthiouracil (PTU) administration and x-irradiation. However, the validation data provided in that report was limited and the method was not made commercially available. Rachofsky *et al.,* (1988) investigated a separate species-specific assay in 79 suspected hypothyroid dogs. Most of the cases were categorised based on their clinical response to T_4 supplementation. However, the results indicated that cTSH estimation either alone or in conjunction with total T_4 measurement was no more predictive of a favourable response to T_4 replacement therapy than a basal total T_4 estimation alone.

Recently Williams *et al.,* (1996) reported the development and laboratory validation characteristics of an IRMA assay for cTSH measurement (Canine TSH IRMA, Diagnostic Products). Canine cTSH was isolated from canine pituitary glands and purified by means of immunoaffinity chromatography using a highly specific monoclonal antibody. The polyclonal rabbit anti-cTSH antibody used in the assay was then produced by immunisation of rabbits with the cTSH and performing immunoaffinity chromatography on the rabbit serum. In the assay, sample cTSH is sandwiched between polyclonal anti-cTSH antibody in the liquid phase, and specific monoclonal anti-cTSH antibody immobilised on the inside of the reaction tubes. The authors reported significant elevations in serum cTSH concentration in six dogs 14 days after experimental radiothyroidectomy. Thyroid status was confirmed by bovine TSH response testing prior to and following radiothyroidectomy. Following T_4 replacement therapy cTSH concentrations decreased and were not significantly different to the baseline values within 14 days of commencing therapy. The results were consistent with expected changes, although actual cTSH concentrations were not provided. Following the development of the cTSH antibody, it has subsequently been commercialised and is now widely available in several kit forms including the IRMA.

Jensen *et al.,* (1996) evaluated the same assay in five hypothyroid dogs, and 11 dogs with various dermatological conditions. Cases were categorised as hypothyroid or euthyroid with NTI by thyroid gland biopsy examination. In addition 13 healthy dogs were studied. Median (range) cTSH concentration was 0.18 μg/L (0.16-0.72) in the hypothyroid dogs which was significantly greater than 0.09 μg/L (0.05-0.53) in the euthyroid dogs with NTI and 0.09 μg/L (0.06-0.34) in the healthy dogs. However, there was considerable overlap of results between the three groups. This may have been related to the classification of cases since the presence of thyroid pathology in itself is not confirmatory of functional

failure and no assessment of thyroid reserve was performed in the study. Furthermore, only a small number of putatively hypothyroid dogs were studied. Jensen *et al.,* (1996) suggested that the reference range cTSH concentrations in the dogs with histopathological evidence consistent with hypothyroidism may have been the result of exhaustion of the pituitary gland's ability to synthesise and release cTSH. Variation in cTSH isoforms, or "microheterogeneity" was also suggested as a possible explanation for the discordant results.

Peterson *et al.,* (1997) evaluated the same cTSH IRMA kit in 150 healthy, 54 hypothyroid and 54 euthyroid dogs with NTI. Bovine TSH response testing was use to differentiate hypothyroidism from euthyroidism with NTI. Median serum cTSH was significantly greater in the hypothyroid (1.5 ng/ml) compared to both clinically healthy (0.19 ng/ml) and euthyroid with NTI (0.28 ng/ml) cases. However "normal" cTSH results, defined from reference limits generated as described above for total T_4, occurred in 13 of 54 (24.1 %) hypothyroid dogs and elevated cTSH concentrations were reported in 4 of 54 (7.6 %) euthyroid dogs with NTI giving a sensitivity and specificity for hypothyroidism of 0.76 and 0.93, respectively. Possible explanations provided by the author for reference range cTSH concentrations in hypothyroid dogs included central hypothyroidism, the presence of undetectable isoforms of cTSH or a variance between the immunological concentrations and biological activity of the cTSH present. No suggested explanation for the increased cTSH concentrations in the euthyroid dogs was provided.

Ramsey *et al.,* (1997) recently investigated an ELISA for cTSH (Canine TSH ELISA, Diagnostic Products). In that study dogs were categorised as either euthyroid, "sick euthyroid", hypothyroid or hypothyroid on non-thyroidal therapy based on clinical signs, total T_4 concentration and TRH stimulation test results. The reference range was defined as the 2.5^{th} to 97.5^{th} percentile values of the results from the euthyroid group. The reference range quoted was 0 to 0.41 ng/ml. Serum cTSH concentrations were elevated in two of 41 euthyroid, five of 16 sick euthyroid, six of nine hypothyroid, and one of six hypothyroid dogs on non thyroidal therapy. However, cases were predominantly classified based on the results of TRH response tests which are known to be very poorly specific for hypothyroidism (Frank, 1996). Ramsey *et al.,* (1997) suggested that down regulation of the pituitary thyrotrophs as a result of chronic hypothyroidism, or alternatively, central hypothyroidism may have been responsible for the discordantly low cTSH concentrations in the putatively hypothyroid group.

Scott-Moncrieff *et al.,* (1998) evaluated the same cTSH IRMA assay as Peterson *et al.,* (1997) in samples from 62 healthy, 16 hypothyroid and 33 euthyroid dogs with various NTI categorised based on TSH response tests. Cases with equivocal response test results were excluded from the study. A reference range for cTSH was generated from the 5th to 95th percentile of results from healthy dogs, and reported to be 0.02 to 0.45 ng/ml. Median (range) cTSH concentrations were 0.10 ng/ml (0.01 to 0.75) in the healthy dogs, 0.47 ng/ml (0.07 to 2.06) in the hypothyroid dogs and 0.18 ng/ml (0.01 to 2.29) in the euthyroid dogs with NTI. Circulating cTSH concentrations were significantly different between each of the three groups. Four of 33 (12 %) euthyroid dogs, and six of 16 (38 %) hypothyroid dogs had unexpectedly increased and decreased cTSH concentrations, respectively. The diagnostic sensitivity and specificity for hypothyroidism were 0.63 and 0.88, respectively. Possible explanations for the discordant cTSH results proposed by the authors included diurnal fluctuation in cTSH, central hypothyroidism, concurrent drug administration, concurrent disease or decreased pituitary secretion of cTSH in cases with chronic hypothyroidism.

From the studies outlined above, it is clear that cTSH measurement alone is neither entirely sensitive or specific for hypothyroidism, and several explanations for discordant results in both euthyroid and hypothyroid dogs have been proposed (Campbell *et al.,* 1996; Jensen *et al.,* 1996; Peterson *et al.,* 1997, Ramsey *et al.,* 1997; Scott-Moncrieff *et al.,* 1998). However, currently there is relatively little data to help identify causes of discordancy. Many normal physiological characteristics, such as the effect of age, breed and sex on cTSH have not yet been evaluated. Bruner, Scott-Moncrieff & Williams, (1998) determined cTSH concentrations, using the same IRMA assay described above, in six healthy dogs, six radiothyroidectomised adult beagle dogs before and during THRT, and in six dogs with spontaneous hypothyroidism every two hours from 8am for 12 hours. Dogs were cateogrised according to clinical signs, circulating endogenous cTSH concentration and total T_4 concentration before and after TRH administration. The cTSH concentrations from the spontaneously hypothyroid dogs were estimated using the chemiluminescent technique (Immulite Canine TSH, Diagnostic Products). Mean (± s.d.) cTSH concentration in the healthy, untreated radiothyroidectomised, treated radiothyroidectomised and spontaneously hypothyroid dogs were 0.11 ± 0.08, 3.31 ± 1.30, 0.08 ± 0.07 and 0.55 ± 0.27 ng/ml respectively. There were no significant differences within groups between time points. All cTSH concentrations from the healthy dogs and treated radiothyroidectomised dogs were within the reference range. All cTSH concentrations from the untreated

radiothyroidectomised were above the reference range. Fifteen of 41 (37 %) samples from the spontaneously hypothyroid dogs had cTSH concentrations greater than the reference range, whereas the remaining 26 (63 %) results were within the reference range. In one dog with naturally occurring hypothyroidism, cTSH concentration was within the reference range at all time points contributing to these results. The authors suggested that central hypothyroidism or the presence of undetectable isoforms of cTSH may have been responsible for the results in this case. None of the spontaneously hypothyroid dogs had cTSH concentrations outwith the reference range at every time point. However, the fluctuations in cTSH concentrations in these dogs was apparently random. The authors concluded that this phenomenon may in part explain the reduced diagnostic sensitivity of cTSH for hypothyroidism and suggested that repeated sampling for cTSH measurement may be of value in suspected hypothyroid dogs.

Amongst the recent reports of the IRMA cTSH assay performance, there is widespread agreement that the LoD of the assay is in the region of 0.01 ng/ml which is inadequate to differentiate between "normal" and low values. This is supported by the data presented in Chapter 3. The current generation of cTSH assays therefore appear unable to confirm central hypothyroidism or be accurately used for therapeutic monitoring purposes.

Thyroglobulin Autoantibodies

The demonstration of circulating autoantibodies to thyroglobulin (TgAb) has been suggested to be useful in the confirmation of thyroiditis in the dog. This subject is discussed in detail in Chapter 7.

Dynamic Thyroid Function Testing

TSH response Test

The most definitive test for the confirmation of hypothyroidism in the dog is the bovine TSH response test (Larsson, 1988; Peterson & Ferguson, 1989; Panciera, 1990b; Ferguson, 1994; Feldman & Nelson, 1996). The principle behind the test is that administration of a supraphysiological dose of exogenous TSH causes maximal stimulation of the thyroid gland. This test therefore provides an assessment of thyroid secretory reserve (Peterson & Ferguson, 1989; Ferguson, 1994). Whilst both drug therapy and NTI can cause a reduction in the thyroid gland's response to exogenous TSH, this test is affected less than any other commonly available technique and accurate identification of

hypothyroidism is possible in most cases (Torres *et al.,* 1991; Feldman & Nelson, 1996; Frank, 1996).

Various protocols and interpretations of TSH response tests have been advocated in the literature. Belshaw & Rijnberk, (1979) compared serum total T_4 concentrations two, four, eight and 10 hours after the intravenous administration of 10 i.u. TSH in 30 dogs and concluded that the eight hour sample gave the highest and most consistently elevated total T_4 concentration. Gosselin *et al.,* (1980) reported maximal total T_3 and total T_4 concentrations eight hours after the intramuscular administration of 0.1 or 0.2 i.u. / 5 lbs body weight bovine TSH but 12 hours after the administration of 1 i.u. / 5 lbs body weight. Lorenz & Stiff, (1980) adopted an unusual protocol whereby blood was collected for serum total T_4 estimation mid-morning and 0.4 i.u. TSH/kg body weight was then administered at 10pm that evening. The following day another sample for total T_4 estimation was collected for comparison. A failure to double serum total T_4 concentration was considered confirmatory of hypothyroidism. Chastain, (1982) recommended sampling before and eight hours after intravenous administration of 5 i.u. or 10 i.u. TSH to dogs less than or greater than 5 kg body weight, respectively. Reimers, Concannon & Cowan, (1982) administered 5 i.u. TSH intravenously and reported peak total T_4 concentrations 4.5 hours later. However in that study no samples were collected after the 4.5 hour sampling time. The same study confirmed simultaneous administration of adrenocorticotropin (ACTH) and TSH had no significant effect on the magnitude of total T_4 increase. Oliver & Waldrop, (1983) administered either 2.5 i.u. or 5 i.u. TSH to dogs intravenously and reported significant increases in total T_4 by three hours after TSH administration with peak total T_4 concentrations occurring after five to seven hours. There was no significant difference in total T_4 results between the two doses used. Kemppainen, Thompson, Lorenz, Munnell & Chakraborty, (1983) administered 0.4 i.u. TSH/kg body weight intravenously and collected serum for total T_4 measurement four hours later. Mean ± s.e.m. increase in total T_4 was 63 ± 6.4 nmol/L in 10 healthy control dogs. Oliver & Held, (1985) evaluated the total T_3 and T_4 response to 0.2 i.u. TSH/kg body weight administered intravenously to 10 clinically healthy dogs. There was a significant increase in total T_3 concentration within 30 minutes of TSH administration and had doubled the baseline value within 60 minutes. The total T_4 concentration was significantly increased within 60 minutes of TSH administration, and had doubled by 90 minutes. Whilst the total T_3 response to TSH administration was therefore more rapid than that that of total T_4, the peak thyroid hormone concentrations were reached four hours post-TSH administration. Whilst the authors suggested that the early

measurement of post-TSH T_3 may therefore assist in a more rapid evaluation of thyroid gland function, such a protocol remains less robust than measurement of peak post-TSH total T_4 concentration. Paradis, Lepine, Lemay & Fontaine, (1991) administered 2 i.u. bovine TSH per dog and collected samples for total T_4 estimation at zero and four hours. The authors recommended that post-TSH total T_4 concentration should increase by at least 24 nmol/L from the basal value, or should be > 45 nmol/L to exclude hypothyroidism. Beale, Keisling & Forster-Blouin, (1992) collected samples for total T_4 estimation before and six hours after the administration of either 2.5 or 5 i.u. TSH to dogs less or greater than 20 kg body weight, respectively. Dogs were considered euthyroid if the post-TSH total T_4 concentration exceeded 38.6 nmol/L. Paradis, Laperriere & Lariviere, (1994) administered 0.1 i.u. TSH/kg body weight to 18 healthy dogs and reported a good total T_4 response at both four and six hours post-TSH. The same study also confirmed that storage of frozen reconstituted TSH at -20°C for up to 200 days had no significant effect on its biological activity in-vivo. Sparkes, Gruffydd-Jones, Wotton, Gleadhill, Evans & Walker, (1995) assessed the response of serum total T_4, total T_3 and free T_4 at two, four, five, six, seven, eight and 10 hours after the intravenous administration of 1 i.u., 3 i.u. and 5 i.u. bovine TSH to six healthy beagles. The method used for "free" hormone estimation was a single step analogue radioimmunoassay and therefore as discussed above, these results can not be considered as an accurate estimation of the genuine free hormone concentration. The peak total T_3 and total T_4 concentrations occurred six hours after 1 i.u. and 3 i.u. TSH and at five and eight hours after 5 i.u. TSH administration, respectively. The total T_4 response to TSH was greater and more consistent than that of total T_3. The optimal time for total T_4 measurement was reported to be six hours post-TSH. Recently Peterson *et al.*, (1997) excluded hypothyroidism if serum total T_4 increased by at least 1.5 times the basal concentration to a value in excess of 25 nmol/L six hours after administration of 0.1 i.u. TSH/kg body weight. Hypothyroidism was confirmed if the post-TSH total T_4 result was less than 20 nmol/L. Scott-Moncrieff *et al.*, (1998) used the same protocol and confirmed hypothyroidism if the post-TSH total T_4 concentration was less than 19.3 nmol/L and excluded it in those with a value greater than 32 nmol/L.

Whilst it is clear that a variety of protocols for both performing and interpreting the TSH response test have been used, the studies outlined above have improved the understanding of the most efficient and effective means of performing the test. Consequently there is now a more consistent approach between workers with 0.1 i.u./kg body weight bovine TSH administered intravenously and sampling for total T_4 at zero and

six hours being generally adopted (Panciera, 1994; Frank, 1996; Peterson *et al.,* 1997; Scott-Moncrieff *et al.,* 1998). This protocol is practical, provides maximal thyroidal stimulation and therefore allows optimal assessment of thyroid reserve. Recommendations on the interpretation of the thyroid hormone results have also become more standardised with values less than approximately 20 nmol/L and greater than approximately 30 nmol/L being confirmatory of hypothyroidism or euthyroidism respectively (Peterson *et al.,* 1997; Scott-Moncrieff *et al.,* 1998). In addition most workers now also require a relative increase in total T_4 results usually in the order of at least 50 % of the basal value. This avoids the potential misclassification of cases which would occur in those hypothyroid individuals with spurious elevated total T_4 concentrations resulting from circulating T_4Ab. Whilst earlier studies have solely recommended the use of a percentage increase in total T_4 irrespective of the absolute hormone concentrations for the confirmation or exclusion of hypothyroidism, this approach is unreliable in cases with very low resting basal total T_4 concentrations in which a small and clinically insignificant increase could be misinterpreted as a "normal" response.

However, there is currently no TSH preparation licensed for diagnostic use in the dog and consequently it is difficult to obtain this product in veterinary practice. In addition, bovine TSH is relatively expensive and therefore its use is generally reserved for research purposes.

TRH Response Test

Because of the cost and availability problems associated with TSH response testing, the TRH response test has been used to confirm hypothyroidism (Jacobs, Lumsden & Willet, 1987; Ramsey *et al.,* 1997). However, the thyroidal response to exogenous TRH is considerably lower and more variable than that obtained with TSH particularly in cases with NTI. Lothrop, Tamas & Fadok, (1984) collected serum for total T_4 estimation immediately before and six hours following the intravenous administration of 2 μg/kg to 200 μg/kg TRH in 31 healthy adult dogs. A significant elevation in total T_4 was reported which peaked at six hours post-TRH. Increased doses of TRH were found to increase the duration but not the peak concentration of total T_4 response. Side effects were reported at all doses greater than 100 μg/kg and included salivation, urination, defaecation, vomition, pupillary constriction, tachycardia and tachypnoea. At a TRH dose of 100 μg/kg, 12 of the healthy animals had a total T_4 response that was less than 1.8 times the basal concentration.

Sparkes *et al.,* (1995) evaluated the total T_4 and total T_3 responses to intravenous administration of 100 μg, 200 μg, 300 μg and 600 μg TRH in six healthy adult beagles. Circulating hormone concentrations were determined immediately before and two, five, six, seven and eight hours after TRH administration. Mean total T_4 concentrations were significantly increased at all sample times after TRH administration irrespective of the dose used although on an individual basis an increase in total T_4 concentrations of less than 50 % basal value was not uncommon. The total T_3 response to TRH was even more variable with few post-injection samples being significantly different to baseline results. Peak total T_4 occurred four hours after 100 μg, and 200 μg TRH and five hours after 300 μg and 600 μg TRH had been given. In the same study, bovine TSH response tests were performed as described previously. The magnitude of response of total T_4 and total T_3 to TSH was significantly greater than that to TRH.

Frank, (1996) compared TSH response tests (total T_4 measurement before and six hours after intravenous administration of 0.1 i.u./kg bovine TSH; maximum dose 5 i.u.) and TRH response tests (total T_4 measurement before and six hours after intravenous administration of 50 μg/kg equine TRH; maximum dose 1mg) in five healthy dogs and 22 dogs with various dermatological complaints. The clinical cases were categorised as either hypothyroid (n=6), or euthyroid with dermatological disease (n=17) based on the TSH response test results. In the group of healthy dogs, post-TRH total T_4 concentrations ranged from 23.8 to 32.5 nmol/L compared to 43.9 to 64.2 nmol/L following TSH administration. Amongst the euthyroid dogs with dermatological disease, post-TRH total T_4 concentrations ranged from 14.6 to 45.0 nmol/L compared to 34.0 to 91.3 nmol/L following TSH administration. The mean response to TSH was significantly greater than that to TRH in both healthy and euthyroid dogs with NTI. Six of 22 euthyroid dogs with dermatological disease and normal total T_4 responses to TSH had no or minimal total T_4 response to TRH administration. There was a numerical although not statistically significant increase in the mean post-TRH total T_4 concentration in these dogs after treatment of their skin disease. The specificity of the TRH response test for hypothyroidism was therefore only 0.73. The smaller and more variable total T_4 response to TRH compared to TSH may have resulted from the effect of other factors which modulate TSH control. Irrespective, the author's conclusion was that compared to the TSH response test, in a clinical setting TRH response testing is an unreliable tool for the confirmation of hypothyroidism and can only reliably be used to exclude the disease.

TSH response to TRH

In humans the sensitivity of the pituitary thyrotrophs to TRH administration tends to be increased during primary hypothyroidism and decreased during NTI (Kaptein, Grieb, Spencer, Wheeler & Nicoloff, 1981; Sumita, Ujike, Namiki, Watanabe, Kawamata, Watanabe & Satoh, 1994). Therefore the TSH response to TRH administration can provide valuable information regarding thyroid status. Since the development of a valid cTSH assay, several studies have also evaluated this test for use in dogs.

Meij *et al.*, (1996) reported the TSH response to 10 μg/kg body weight TRH administered intravenously in eight healthy beagles. Mean ± s.e.m. resting cTSH concentrations were 0.14 ± 0.02 ng/ml (range 0.07 to 0.27 ng/ml) which was significantly lower than that of the 10 minute post-TRH cTSH concentration of 1.26 ± 0.22 ng/ml. A slightly reduced TSH response to TRH was observed when TRH was administered concurrently with other hypothalamic releasing hormones, namely corticotrophin releasing hormone, growth hormone releasing hormone and gonadotrophin releasing hormone. That study provided a basis for further assessment of the responsiveness of the pituitary gland to TRH in states of thyroidal and non thyroidal disease.

Ramsey & Herrtage, (1997) examined the cTSH response to TRH in an unspecified number of dogs from approximately 50 which were being investigated for hypothyroidism. That study reported no clear distinction in response between the hypothyroid and euthyroid dogs. The classification of cases as either hypothyroid or euthyroid with NTI relied on the results of TRH response tests and therefore the classification of cases is questionable.

Scott-Moncrieff & Nelson, (1998) investigated the cTSH response to TRH administration in 13 healthy, 20 hypothyroid and 18 euthyroid dogs with NTI. Hypothyroidism or euthyroidism was confirmed based on TRH response tests, endogenous cTSH concentration and response to thyroid hormone replacement therapy. The cTSH concentration 30 minutes following TRH administration in the hypothyroid group (median 1.45; range, 0.17-4.2 ng/ml) was significantly greater than in the euthyroid group (median 0.57; range, 0.17-1.6 ng/ml), but not significantly different to the healthy dogs (median 0.69; range, 0.4-1.6 ng/ml). As a percentage of the basal cTSH concentration, the increase in cTSH was significantly lower in the hypothyroid dogs (median 24 %; range, -21-134 %) compared to both the euthyroid (median 167 %; range, 69-1800 %) and healthy (median 207 %; range, 25-2200 %) groups. Using a > 100 % increase in cTSH concentration after TRH administration as a cut-off for the exclusion of hypothyroidism, the diagnostic sensitivity, specificity and accuracy were 0.85, 0.94 and 0.90, respectively. The authors

concluded that whilst the test did discriminate between hypothyroid and euthyroid dogs, it was of little advantage over a basal cTSH and either total or free T_4 estimation. The reason for the differences in cTSH response to TRH between dogs and man is currently unclear.

Therapeutic Trial

The confirmation of a diagnosis of hypothyroidism by demonstrating an appropriate clinical response to T_4 replacement therapy, holds initially at least, considerable appeal. This approach is simple and rapid and avoids the problems associated with laboratory testing. However, T_4 therapy can be associated with non-specific clinical responses which can create the impression of a genuine response to treatment and a failure to respond to supplementation may result from factors other than an erroneous diagnosis.

Gunaratnam, (1986) determined the rate of hair growth at various sites in healthy crossbreed dogs and found a significant increase in the rate of growth following therapy with either oral (20 μg/kg/day) or topical L-thyroxine preparations. Clearly the demonstration of a clinical response to T_4 therapy is therefore not in itself confirmatory of hypothyroidism. Considerable individual variation also exists in the pharmacokinetics of thyroid hormones in vivo, and a suitable dosage for one dog may not be appropriate for another individual. This may be misinterpreted as a failure to respond to therapy. This is discussed in Chapter 6. Circulating concentrations of thyroid hormones may vary by as much as four-fold in different dogs receiving similar doses of thyroxine, whilst levothyroxine doses between 11-44 μg/kg/day have been required to obtain optimal circulating concentrations in different individuals (Nachreiner, Refsal, Ravis, Hauptman, Rosser & Pedersoli, 1993). The same study demonstrated that the pharmacokinetics of T_4 were fairly constant within an individual, but varied considerably between individual dogs.

A marked drawback in the use of T_4 therapy as a diagnostic tool, results from the affect that the medication will have on subsequent tests of thyroid function. Panciera *et al.*, (1989) studied the effect of exogenous T_4 therapy at a dose recommended for treatment of hypothyroidism in 10 euthyroid beagles. Severely suppressed total T_4 response to exogenous TSH administration occurred within four weeks of starting therapy and remained so until at least four weeks after the cessation of treatment. Inappropriate T_4 replacement therefore delays and confuses any subsequent tests of thyroid status.

In the situation where no other diagnostic options are available, it has been recommended that T_4 replacement therapy be used as a diagnostic tool under strict conditions. Ferguson, (1994) suggested that an objective criterion by which the success of

the therapy will be judged (such as regrowth of at least 50 % hair coat) and a time limit over which the dog will be re-evaluated be used. In the case of an appropriate clinical response, it was suggested that therapy should then be stopped and the case monitored for a return of the clinical signs. Resolution of these signs following re-institution of thyroid hormone therapy could then be considered confirmatory of the diagnosis. The main difficulty with his approach in practice is the understandable reluctance of dog owners to stop therapy in a responding animal.

The changes in thyroid hormone metabolism associated with NTI are presumed to be protective mechanisms aimed at reducing metabolic requirements and avoiding catabolism of tissue reserves. Over-riding this mechanism with T_4 supplementation may therefore be detrimental and should be avoided. Brent & Hershman, (1986) demonstrated no improvement in clinical outcome in patients with severe NTI following T_4 replacement and Little, (1985) demonstrated an increase in mortality in rats with experimentally induced NTI. Although equivalent canine studies have not yet been reported, it seems prudent to ensure a confident diagnosis of hypothyroidism whenever possible prior to instituting thyroid hormone replacement therapy.

4.2 INTRODUCTION

There is no doubt that the single most reliable method for confirming canine hypothyroidism is the bovine TSH response test. However, this test is not suitable for widespread use in general practice. A range of commercial diagnostic tests including kits for cTSH, free T_4 by dialysis and TgAb have recently become available. However, there is conflicting evidence regarding the diagnostic accuracy of these newer tests (Jensen *et al.*, 1996; Peterson *et al.*, 1997; Ramsey *et al.*, 1997; Scott-Moncrieff *et al.*, 1998). There is also little data to indicate causes of apparently discordant results. The objectives of this study were to determine if a single test or combination of tests suitable for analysis in a single blood sample could be used in the diagnosis of hypothyroidism with an accuracy approaching that of the "gold standard" bovine TSH response test, and identify the optimal testing strategy in dogs with suspected hypothyroidism.

4.3 MATERIALS AND METHODS

Case Material

The case material comprised a series of 140 dogs referred to the DVCS for investigation of clinical signs or routine laboratory features consistent with adult onset spontaneous hypothyroidism as described in Chapters 2 and 5. Dogs were classified as either euthyroid or hypothyroid based on the results of TSH response tests as described in Chapter 2.

Assays

Serum total T_4, total T_3, free T_4 and cTSH were measured using commercially available assays as described in Chapter 2.

Receiver Operating Characteristic Curves and Differential Positive Rate Analysis

The diagnostic performance of the total T_4, total T_3, free T_4 and cTSH assays both individually and for some parameters in combination with each other, were evaluated by DPR and ROC curve analysis which were calculated and graphically represented as described in Chapter 2. Specifically, DPR analysis was used to determine optimal cut-off values, and ROC curve analysis to compare the performance between tests.

Statistical Analyses

A Mann-Whitney U test was used for comparison of hormone results between hypothyroid and euthyroid groups. For all statistical analyses, results less than the LoD of an assay were arbitrarily assigned a value equal to the LoD.

4.4 RESULTS

Case Material

Hypothyroidism was confirmed in 52 dogs (mean age 7.6; range 3-13 years) containing 23 male (5 neutered) and 29 female (15 neutered) animals. Results of the TSH response tests were equivocal in three of these cases. Further details of these animals are provided in Appendix 27. Mean ± s.d. post TSH total T_4 concentration in the remaining 49 dogs was 8.2 ± 5.5 nmol/L. Euthyroidism was confirmed in 88 dogs (mean age 7.3; range 1-14.5 years) of which 49 were male (10 neutered) and 39 female (22 neutered). Results of the TSH response tests were equivocal in nine of these cases. Further details of these animals are provided in Appendix 27. Mean ± s.d. post TSH total T_4 concentration in the remaining 79 dogs was 65.3 ± 32.1 nmol/L. No adverse reactions to intravenous bovine TSH administration were recognised in any of the dogs.

Hormone Results

The results of the hormone analyses from the hypothyroid and euthyroid groups are summarised in Tables 2 and 3, respectively. Individual case data are provided in Appendices 29 and 30.

	Total T_4 (nmol/L)	**Total T_3 (nmol/L)**	**Free T_4 (pmol/L)**	**cTSH (ng/ml)**
Mean	7.3	1.50	4.86	2.03
s.d.	4.2	0.75	3.10	2.31
Median	4.7	1.32	3.22	1.38
25th Percentile	4.7	1.03	3.22	0.73
75th Percentile	10.2	1.79	4.93	2.62
Minimum	4.7	0.25	3.22	0.01
Maximum	21.2	23.49† (3.88)	12.39	12.3

Table 2. Circulating concentrations of total T_4, total T_3, free T_4 and cTSH obtained from 52 hypothyroid dogs.

† Total T_3 concentration of 23.49 nmol/L was attributed to the presence of T_3Ab and was not used in calculations. The next highest result in this group was 3.88 nmol/L.

	Total T_4 (nmol/L)	Total T_3 (nmol/L)	Free T_4 (pmol/L)	cTSH (ng/ml)
Mean	21.9	2.33	16.32	0.41
s.d.	11.2	0.75	8.94	0.45
Median	20.1	2.38	13.84	0.27
25^{th} Percentile	13.4	1.83	10.42	0.13
75^{th} Percentile	27.5	2.82	21.29	0.54
Minimum	4.7	0.78	3.22	0.01
Maximum	60.5	4.71	51.25	2.65

Table 3. Circulating concentrations of total T_4, total T_3, free T_4 and cTSH results obtained from 88 euthyroid dogs.

Total T_4

DPR data for total T_4 are illustrated in Figure 2. The ROC curve of total T_4 results is illustrated in Figure 3.

Median basal serum total T_4 was significantly ($p<0.0001$) lower in the hypothyroid compared with the euthyroid dogs. DPR analysis indicated an optimal cut-off value of 7.6 nmol/L for confirmation of hypothyroidism resulting in a sensitivity, specificity and DPR of 0.71, 0.97 and 0.68, respectively. The DPR remained relatively constant across a range of values from approximately 6 to 15 nmol/L indicating that a cut-off anywhere within this range would provide similar overall test performance. Because basal total T_4 measurement is generally used as a screening test for hypothyroidism, a cut-off value associated with a greater diagnostic sensitivity was considered more desirable. Examination of the range of DPR values identified 14.8 nmol/L as a suitable cut-off value and this was selected for the confirmation of hypothyroidism. This value was associated with a sensitivity, specificity and DPR of 0.96, 0.70 and 0.67, respectively. The area under the ROC curve was 0.919.

Two hypothyroid dogs had total T_4 concentrations greater than 14.8 nmol/L. One of these cases had an elevated cTSH value (6.3 ng/ml), subnormal free T_4 (6.66 pmol/L) concentration and negligible response to TSH administration. The remaining case had an equivocal total T_4 response to TSH administration and had reference range free T_4 (12.39

pmol/L) and cTSH (0.57 ng/ml) concentrations, but hypothyroidism was supported by histopathological evidence of autoimmune thyroiditis. This case had been presented to the DVCS for investigation of musculoskeletal illness, not considered to be related to the hypothyroidism, during which time the evidence of thyroid disease became apparent. However the case was almost certainly in the very earliest stages of hypothyroidism and it is unlikely that this cases would have been presented to a veterinary surgeon for investigation of signs primarily related to hypothyroidism at the time the diagnosis was confirmed.

Twenty six euthyroid dogs had subnormal total T_4 concentrations. Of these cases, cTSH was within the reference range in 21 and was increased in five. Free T_4 concentration was measured in four of these five dogs and found to be in the euthyroid range in each case. Free T_4 was measured in 22 of the 26 euthyroid cases with subnormal total T_4, and was subnormal in only five. The cTSH concentration was within the reference range in all five of these dogs.

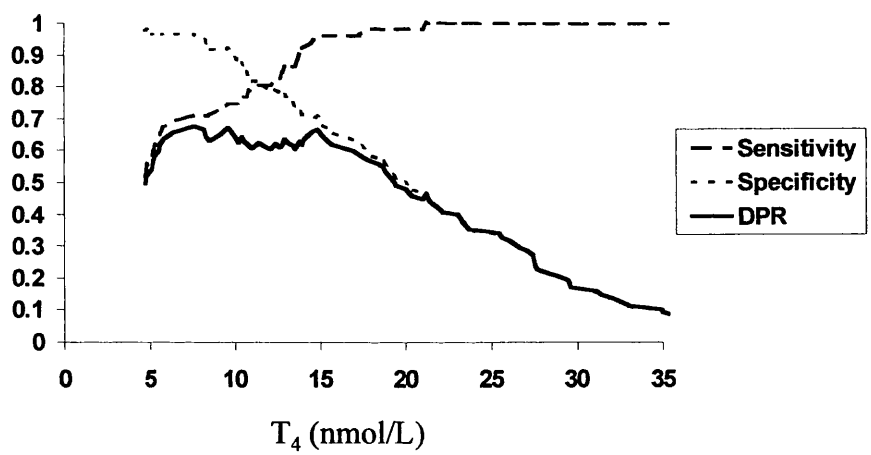


Figure 2. Differential positive rate (DPR) curve demonstrating the change in diagnostic sensitivity, specificity and DPR of total T_4 for hypothyroidism across a range of possible diagnostic cut-off values.

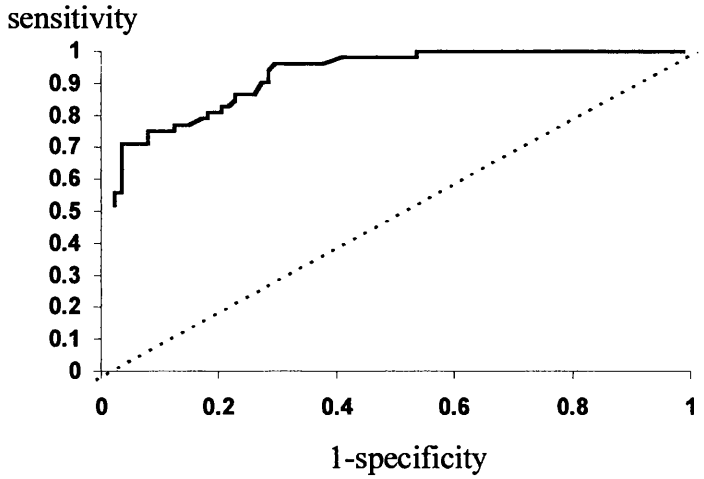


Figure 3. Receiver operating characteristic (ROC) curve for total T_4 in distinguishing hypothyroid from euthyroid dogs. See Figure 1 for interpretation.

Total T_3

DPR data for total T_3 are illustrated in Figure 4. The ROC curve of total T_3 results is illustrated in Figure 5.

Median serum total T_3 was significantly ($p<0.0001$) lower in the hypothyroid compared with the euthyroid dogs. DPR analysis indicated an optimal cut-off value of 1.77 nmol/L for confirmation of hypothyroidism resulting in a sensitivity, specificity and DPR of 0.76, 0.80 and 0.56, respectively. The area under the ROC curve was 0.805.

Eleven hypothyroid dogs had total T_3 concentration greater than 1.77 nmol/L. One of these results was extremely elevated (23.49 nmol/L) consistent with interference by T_3 autoantibodies, an assumption which was supported by the presence of TgAb in this case (Chapter 7). This total T_3 result was excluded from all further analyses. Nine, nine and seven of the 11 dogs had total T_4, free T_4 and cTSH results within the "hypothyroid" ranges, respectively.

Fifteen euthyroid dogs had a total T_3 concentration less than 1.77 nmol/L. Of these cases, basal total T_4 was normal in nine, and free T_4 and cTSH were normal in 14.

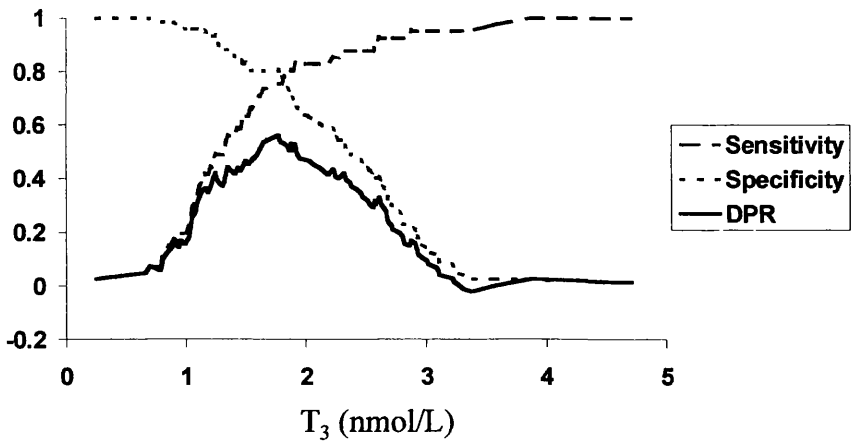


Figure 4. Differential positive rate (DPR) curve demonstrating the change in diagnostic sensitivity, specificity and DPR of total T_3 for hypothyroidism across a range of possible diagnostic cut-off values.

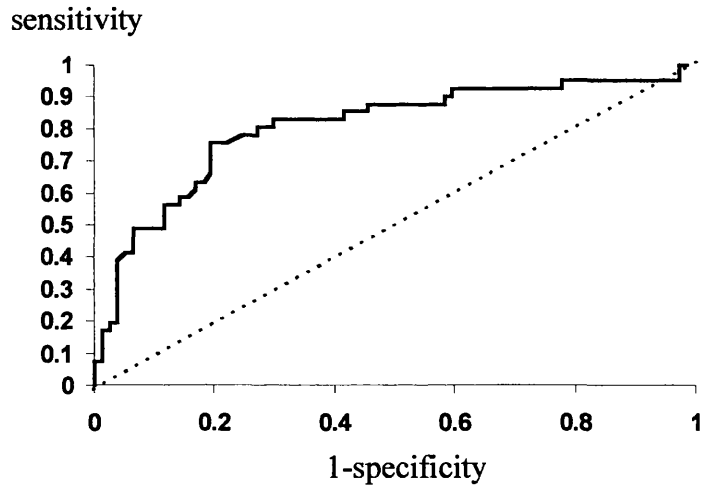


Figure 5. Receiver operating characteristic (ROC) curve for total T_3 in distinguishing hypothyroid from euthyroid dogs. See Figure 1 for interpretation.

Free T_4

DPR data for free T_4 are illustrated in Figure 6. The ROC curve of free T_4 results is illustrated in Figure 7.

Median serum free T_4 was significantly ($p<0.0001$) lower in the hypothyroid compared with the euthyroid dogs. DPR analysis indicated an optimal cut-off value of 6.66 pmol/L for confirmation of hypothyroidism resulting in a sensitivity, specificity and DPR of 0.81, 0.93 and 0.74, respectively. The area under the ROC curve was 0.933.

Eight hypothyroid dogs had free T_4 concentrations greater than 6.66 pmol/L. These included the five dogs with the greatest post-TSH total T_4 concentration amongst the hypothyroid group. Of these eight dogs, total T_4 was depressed and cTSH was concurrently increased in seven. The remaining eighth case was the dog with reference range total T_4 concentration and musculoskeletal disease referred to previously. Total T_3 was estimated from seven cases with hypothyroidism and normal free T_4 concentration and was subnormal in five of them.

Six euthyroid dogs had free T_4 concentrations less than 6.66 pmol/L. These included one dog with hyperadrenocorticism, three who had recently received various medications including glucocorticoids, phenobarbitone and non-steroidal anti-inflammatory drugs (NSAID), and two dogs with testicular neoplasia. Total T_4 results were within the hypothyroid range in five of these cases. Circulating cTSH results were within the euthyroid range in all cases. Total T_3 results were normal in all but one dog.

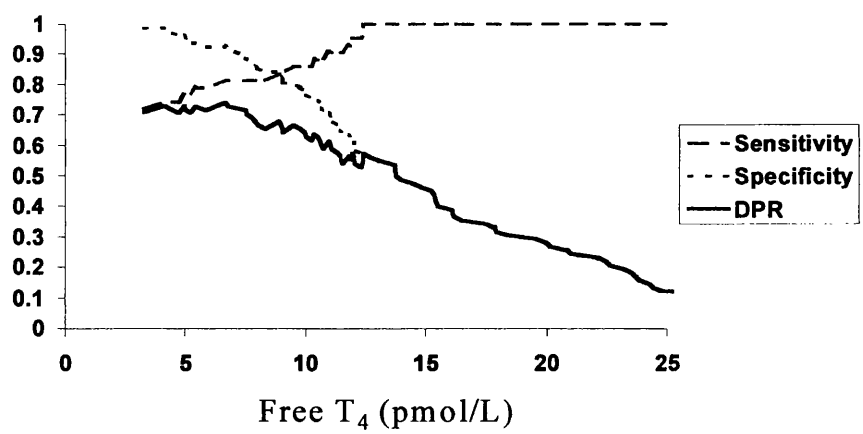


Figure 6. Differential positive rate (DPR) curve demonstrating the change in diagnostic sensitivity, specificity and DPR of free T_4 for hypothyroidism across a range of possible diagnostic cut-off values.

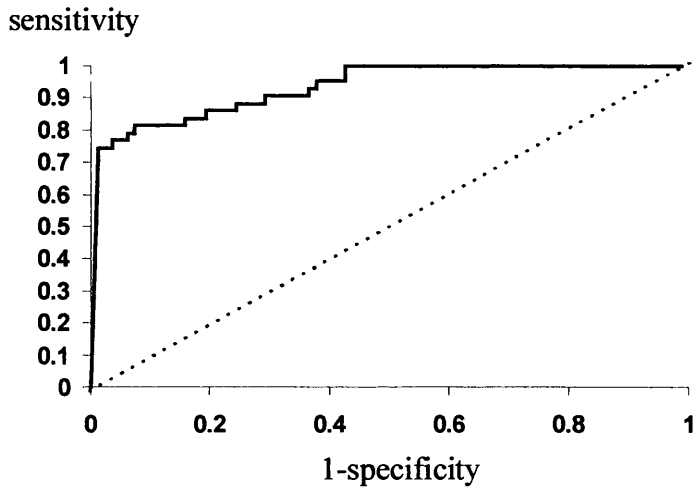


Figure 7. Receiver operating characteristic (ROC) curve for free T_4 in distinguishing hypothyroid from euthyroid dogs. See Figure 1 for interpretation.

Endogenous TSH

DPR data for cTSH are illustrated in Figure 8. The ROC curve of cTSH results is illustrated in Figure 9.

Median serum cTSH was significantly ($p<0.0001$) greater in the hypothyroid compared with the euthyroid dogs. DPR analysis indicated an optimal cut-off value of 0.69 ng/ml for confirmation of hypothyroidism resulting in a sensitivity and specificity and DPR of 0.79, 0.82 and 0.61, respectively. The area under the ROC curve was 0.816.

Eleven of the 52 hypothyroid dogs had cTSH results less than 0.69 ng/ml. Concurrent illness was identified in eight of these cases and included diabetes mellitus (n=2), hyperadrenocorticism (n=1), encephalitis (n=1), orthopaedic disease (n=1), dilated cardiomyopathy (n=1), idiopathic hyperchylomicronaemia (n=1) and gastrointestinal disease (n=1). Of the remaining three cases, the cTSH result approached the LoD of the assay, consistent with central hypothyroidism, in only one case (0.02 ng/ml). Further studies performed in this case are detailed in Appendix 28. and were consistent with a possible diagnosis of central hypothyroidism. No known cause of the reference range cTSH concentration was identified in the remaining two individuals. In one of these two dogs free T_4 was undetectable. No additional diagnostic test results were available in the solitary remaining case. Free T_4 was measured in eight of the hypothyroid cases with discordant

cTSH concentrations and found to be below the assay LoD in seven of them. Total T_3 values were also measured in eight of the dogs, and were in the hypothyroid range in four.

Sixteen euthyroid dogs had cTSH concentrations greater than 0.69 ng/ml. Five of these cases were recovering from a known NTI and three had a history of recent use of sulphonamide-containing medication. Basal total T_4 concentration was greater than 14.8 nmol/L in 11 of the discordant euthyroid cases. In two of the remaining five dogs total T_4 and cTSH returned to normal at a later date including one dog who was recovering from hypoadrenocorticism at the time of testing. In the remaining three cases no cause of the elevated cTSH was identified. Free T_4 was measured in 15 of the 16 euthyroid cases with elevated cTSH values, and was within the euthyroid range in all of them. Total T_3 was measured in 13 of the discordant euthyroid cases and was within the euthyroid range in 12 of them.

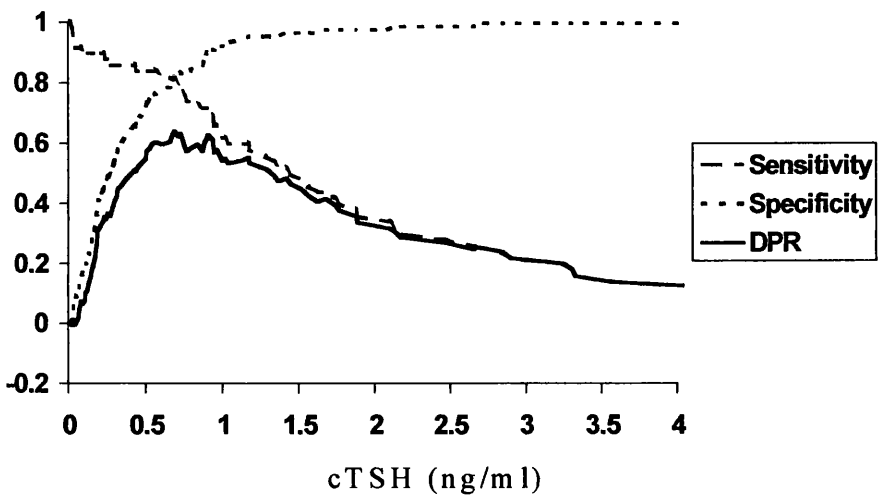


Figure 8. Differential positive rate (DPR) curve demonstrating the change in diagnostic sensitivity, specificity and DPR of endogenous thyrotropin (cTSH) for hypothyroidism across a range of possible diagnostic cut-off values.

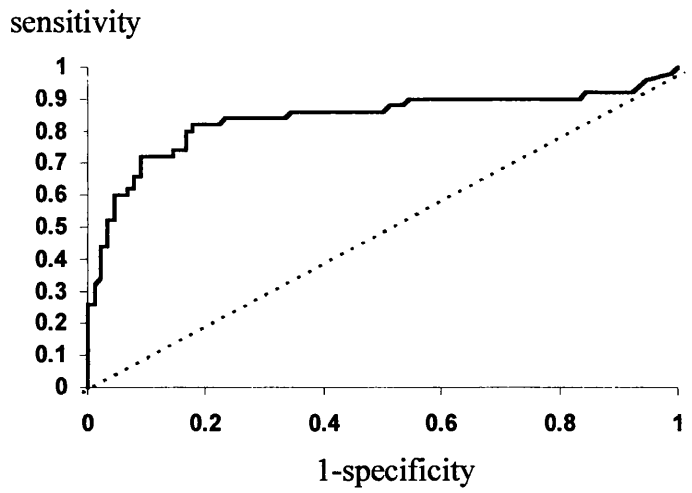


Figure 9. Receiver operating characteristic (ROC) curve for endogenous thyrotropin (cTSH) in distinguishing hypothyroid from euthyroid dogs. See Figure 1 for interpretation.

Thyroxine to Thyrotropin ratios

DPR data for the total T_4 to cTSH ratio are illustrated in Figure 10. The ROC curve of the total T_4 to cTSH ratio results is illustrated in Figure 11.

Median total T_4 to cTSH ratio was significantly ($p<0.0001$) greater in the euthyroid compared with the hypothyroid dogs. DPR analysis indicated an optimal cut-off value of 13.1 for the confirmation of hypothyroidism resulting in a sensitivity, specificity and DPR of 0.75, 0.97 and 0.72, respectively. The area under the ROC curve was 0.893.

The use of a ratio, and the consequent mathematical nature of the results obtained in cases (i.e. a multiplication occurs when dividing by a denominator less than one) was responsible for most of the discordant total T_4 to cTSH results in hypothyroid dogs in this study. Thirteen hypothyroid dogs had unexpectedly increased total T_4 to cTSH ratios and circulating cTSH concentration was less than 1 ng/ml in all these cases, and less than the cut-off for hypothyroidism in 11. Mean (± s.d.) cTSH concentration in the discordant dogs was (0.29 ± 0.32 ng/ml) compared with 2.62 (± 2.39 ng/ml) in remaining hypothyroid cases.

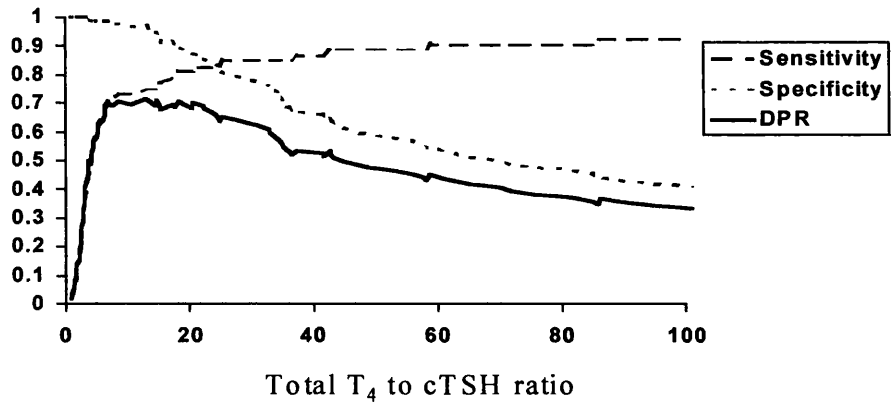


Figure 10. Differential positive rate (DPR) curve demonstrating the change in diagnostic sensitivity, specificity and DPR of the total T_4 to endogenous thyrotropin (cTSH) ratio for hypothyroidism across a range of possible diagnostic cut-off values.

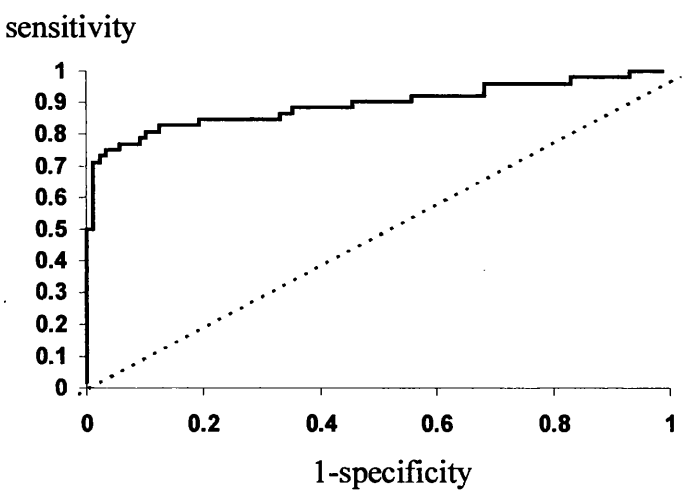


Figure 11. Receiver operating characteristic (ROC) curve for total T_4 to endogenous thyrotropin (cTSH) ratio in distinguishing hypothyroid from euthyroid dogs. See Figure 1. for interpretation.

DPR data for free T_4 to cTSH ratios are illustrated in Figure 12. The ROC curve of free T_4 to cTSH ratio results is illustrated in Figure 13.

Median free T_4 to cTSH ratio was significantly ($p<0.0001$) greater in the euthyroid compared with the hypothyroid dogs. DPR analysis indicated an optimal cut-off value of 13.2 for the confirmation of hypothyroidism resulting in a sensitivity, specificity and DPR of 0.84, 0.90 and 0.74, respectively. The area under the ROC curve was 0.912.

Seven hypothyroid dogs had unexpectedly increased free T_4 to cTSH ratios. As occurred with the total T_4 to cTSH ratios, this was generally due to unusually decreased cTSH values. This mathematical complicating effect occurred despite undetectable free T_4 concentrations in five of the seven cases.

Eight euthyroid dogs had unexpectedly decreased free T_4 to cTSH ratios. Again, these typically resulted from discordant cTSH values, since six of the eight cases had cTSH concentrations within the hypothyroid range.

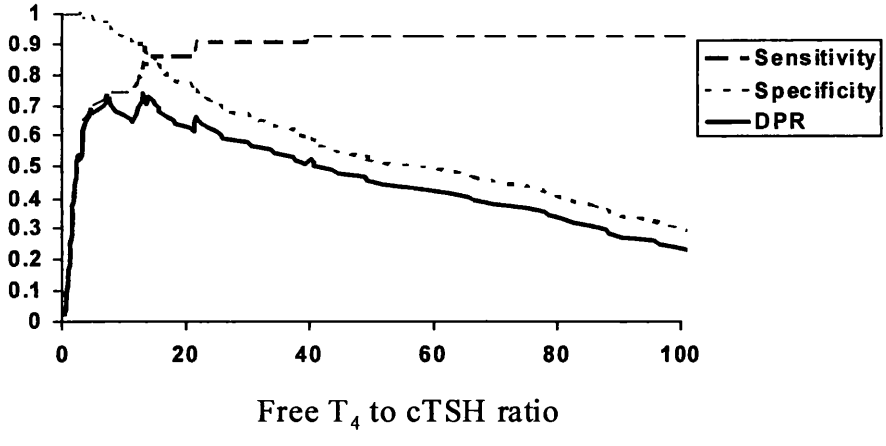


Figure 12. Differential positive rate (DPR) curve demonstrating the change in diagnostic sensitivity, specificity and DPR of the free T_4 to endogenous thyrotropin (cTSH) ratio for hypothyroidism across a range of possible diagnostic cut-off values.

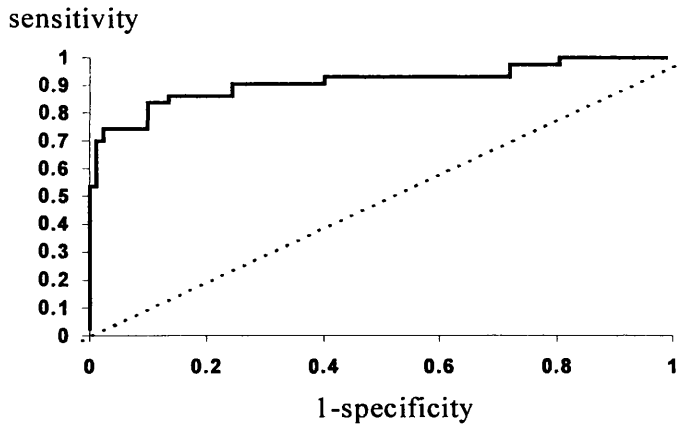


Figure 13. Receiver operating characteristic (ROC) curve for free T_4 to endogenous thyrotropin (cTSH) ratio in distinguishing hypothyroid from euthyroid dogs. See Figure 1 for interpretation.

The diagnostic performance of total T_4, total T_3, free T_4 and cTSH estimation, when used alone and in combination for the diagnosis of hypothyroidism are summarised in Table 4.

	Total T_4 (nmol/L)	**Total T_3 (nmol/L)**	**Free T_4 (pmol/L)**	**cTSH (ng/ml)**	**Total T_4 to cTSH ratio**	**Free T_4 to cTSH ratio**
Cut-off	14.8	1.77	6.66	0.69	13.1	13.2
Sensitivity	0.96	0.76	0.81	0.79	0.75	0.84
Specificity	0.70	0.80	0.93	0.82	0.97	0.90
DPR	0.67	0.56	0.74	0.61	0.72	0.74
Area under ROC curve	0.919	0.805	0.933	0.816	0.893	0.912

Table 4. Diagnostic cut-off, sensitivity, specificity, differential positive rate (DPR) and area under the receiver operating characteristic (ROC) curve for serum total T_4, total T_3, free T_4 and cTSH concentrations, and total and free T_4 to cTSH ratios.

4.5 DISCUSSION

Ideally a diagnostic test would have a sensitivity and specificity both equal to one, i.e. no false positive or false negative results. However, in practice this is rarely the case and the actual values depend largely on the diagnostic cut-off chosen for confirmation or exclusion of disease. Altering the cut off in one direction will change the sensitivity and specificity whilst moving it in the other direction will have the opposite effect. For example decreasing the diagnostic cut-off point of a test in which high results are considered abnormal will increase the diagnostic sensitivity but decrease the specificity. However increasing the cut-off for the same test or decreasing the cut-off for a test in which low results are abnormal will decrease the test sensitivity but increase the specificity. In practice the chosen cut-off point depends largely on the relative requirements for either a sensitive or a specific test.

As is evident from the reviewed literature, most cut-off values are selected either by use of laboratory reference limits or by generation of a "normal range" from apparently healthy dogs. However, as discussed in Chapter 5, such values are rarely the most appropriate cut-off's to use. A simple mathematical method for calculation of the most accurate cut-off is achieved by use of DPR analysis. The DPR equals the sensitivity minus (1-specificity) and can be calculated at each possible cut-off value for a given test. The cut-off with the highest DPR gives the best overall test performance. Alternatively, the DPR can be used to identify a cut-off which will give a test with the desired values for either diagnostic sensitivity or specificity. For example if the test is to be used primarily as a screening test then diagnostic sensitivity is of most importance and an appropriate value can be selected. Alternatively if confident confirmation of disease is more important, then DPR analysis can be used to identify a cut-off value with a suitably high diagnostic specificity. It should be clear that the DPR is essentially another method of representing the data used for ROC curve generation. The greatest DPR value corresponds to the point on the ROC curve nearest to the top left hand corner of the graph. DPR analysis is of most use in the selection of optimal cut-off values whereas comparison of several diagnostic tests is best achieved by ROC curve generation.

This study has confirmed the applicability of DPR and ROC curve analysis to the study of thyroid disease in the dog. DPR analysis proved to be a valuable and flexible statistical tool in the categorisation of cases and optimisation of the diagnostic performance of the thyroid tests studied. The techniques assisted in the identification of appropriate cut-off values for confirmation of hypothyroidism and assisted in the comparison of the performance of the various tests investigated. The clinical utility of DPR analysis in

particular, was exemplified by its identification of a suitable cut-off value for total T_4 concentration, following selection of the desired diagnostic performance criteria. A technical limitation of the methods currently available for DPR analysis, is that only cut-off values actually obtained from at least one individual in the study are available for selection as the 'optimal' cut-off. Currently there is no software designed to allow interpolation of the DPR at values in between those seen in study cases. The production of such a computerised model would enhance this already valuable technique.

This study has confirmed that circulating concentrations of total T_4, free T_4 measured by equilibrium dialysis and total T_3 are usually decreased, and cTSH usually increased, in spontaneous canine hypothyroidism. A high diagnostic sensitivity of total T_4 estimation for hypothyroidism was confirmed, supporting the results of previous studies (Belshaw & Rijnberk, 1979; Kaelin, Watson & Church, 1986; Peterson *et al.,* 1997). However, as exemplified by the similar DPR values across a range of possible cut-off values, this could be manipulated at the expense of specificity depending on the required test characteristics without significant loss of over-all performance. This feature of total T_4 measurement in the diagnosis of hypothyroidism has not been reported previously. Approximately one third of the euthyroid dogs with NTI had a depressed total T_4 concentration confirming the poor specificity of this analyte for hypothyroidism. This supports the results of previous studies (Lorenz & Stiff, 1980; Larsson, 1988; Peterson *et al.,* 1997).

Previous studies have reported poor discriminatory ability of total T_3 in the differentiation of hypothyroidism from NTI and consequently total T_3 estimation is now uncommonly performed in dogs (Nelson *et al.,* 1991; Miller *et al.,* 1992). The results of this study confirm the poor overall performance of total T_3 compared to the other analytes as demonstrated by the low corresponding DPR and area under the ROC curve. However, the results demonstrate that in contrast to humans, total T_3 is less frequently depressed in dogs with NTI than is total T_4. This is similar to previous reports including the recent study by Peterson *et al.,* (1997) who used the same diagnostic criteria for case allocation as the present study. Of the 15 euthyroid dogs with depressed circulating total T_3 values in the present study, serum total T_4 was normal in nine suggesting that hypothyroidism was highly unlikely. In addition, only one each of the remaining six dogs had abnormal free T_4 or cTSH concentrations. Consequently an erroneous diagnosis of hypothyroidism could generally be avoided by concurrent examination of additional indices of thyroid function. In

contrast, the hypothyroid dogs with total T_3 values greater than 1.77 nmol/L generally had abnormal total T_4, free T_4 and cTSH results.

Free T_4 measured by equilibrium dialysis was the single most accurate test investigated supporting the findings of Peterson *et al.,* (1997). This was exemplified by the greater area under the ROC curve, and particularly by the higher optimal DPR for free T_4 compared to total T_4, total T_3 or cTSH measurement. Normal free T_4 results were obtained from eight hypothyroid dogs. Interestingly these included the five individuals with the highest post-TSH total T_4 concentrations. The post-TSH total T_4 concentration is an indicator of maximal thyroid functional reserve and therefore reflects the extent of thyroidal destruction. The normal circulating free T_4 concentration in these five dogs may be consistent with an attempt to maintain thyroid function in the face of impending failure. Subnormal free T_4 was an uncommon occurrence in the euthyroid dogs, confirming the high diagnostic specificity of this analyte for hypothyroidism. Drug preparations including glucocorticoids, phenobarbitone and non-steroidal anti-inflammatory therapies were being administered to some of these cases and may have been responsible for the decreased free T_4 results. Interestingly, two of the six euthyroid dogs with subnormal free T_4 values had testicular tumours. The role of these in the free T_4 results obtained is unclear. Although oestrogen concentration was not measured in these cases, hyperoestrogenism has been previously shown to have only minimal effect on thyroid function (Gosselin *et al.,* 1980). This study has confirmed the considerably greater clinical value of estimation of free T_4 using a dialysis method rather than the commonly used "analogue" immunoassays (Nelson *et al.,* 1991). This is similar to the conclusions of previous reports comparing available free T_4 methodologies in human samples (Ekins, 1993).

This study clearly demonstrated the ability of circulating cTSH estimation to assist in the differentiation of hypothyroidism from NTI. Whilst the test is therefore a very useful tool in the investigation of canine hypothyroidism, inappropriately elevated or depressed cTSH results did occur in 18 and 21 % of euthyroid and hypothyroid cases, respectively. The specificity of cTSH estimation for hypothyroidism reported in this study may have been artefactually reduced slightly since a few of the dogs were referred following identification of abnormal circulating cTSH concentrations. This inherent bias will tend to increase the percentage of false-positive cTSH results obtained.

The most common apparent causes of discordant cTSH results in euthyroid dogs in this study included recovery from a known NTI and the recent use of sulphonamide-containing medication supporting the results of previous studies (Spencer, Eigen, Shen,

Duda, Qualls, Weiss & Nicoloff, 1987; Campbell *et al.,* 1996). This results of this study therefore indicate that a thorough history taking, and as far as possible the exclusion of NTI form a crucial part of the investigation of suspect hypothyroidism. It is recommended that these are performed prior to diagnostic testing. Of the remaining discordant euthyroid cases, cTSH returned to normal in three of the dogs from which further samples were obtained and therefore follow-up cTSH estimations may be useful in some cases when the diagnosis in an individual remains in doubt.

In humans, "subclinical" or "compensating" hypothyroidism, whereby TSH concentration increases in the early stages of thyroid dysfunction to maintain normal circulating total thyroid hormone concentration, is recognised. In these individuals, circulating TSH concentrations are "inappropriately" increased but are a good predictor of the future development of hypothyroidism. Such a phenomenon has also been proposed in dogs (Graham *et al.,* 1998). In theory, this may have been a cause of some of the discordant cTSH values which occurred in euthyroid dogs in the present study. Investigation of this would require long-term follow up of suspect cases and was not performed. However, many humans with "compensating hypothyroidism" are identified during routine screening tests and are not necessarily clinically unwell. This is in contrast to the dogs in this study, all of which had clinical abnormalities consistent with hypothyroidism. At present, health screening tests are rarely performed in dogs. Consequently, if dogs do undergo a period of "subclinical" hypothyroidism prior to the development of clinical hypothyroidism, there is no reason to expect those cases to be blood sampled until the subclinical phase has ended, and the clinical abnormalities developed. Alternative diagnoses were reached in all euthyroid cases in the present study suggesting that "compensating hypothyroidism" was not responsible. Furthermore it is unclear why dogs with compensating hypothyroidism, and therefore by definition "normal" circulating thyroid hormone concentrations would develop clinical signs of thyroid dysfunction. Nevertheless this may be an area worthy of future study as health screening and preventative medicine become more routinely performed in veterinary medicine.

Possible causes of discordant cTSH results in hypothyroid dogs have been proposed including undetectable isoforms of cTSH and central hypothyroidism (Jensen *et al.,* 1996; Peterson *et al.,* 1997; Ramsey *et al.,* 1997; Scott-Moncrieff *et al.,* 1998). The results of this study suggest that spontaneous central hypothyroidism is uncommon since cTSH concentrations at or near the assay LoD were uncommon in the hypothyroid dogs. Central hypothyroidism therefore does not appear to be a frequent cause of discordant cTSH

concentrations. The possibility of undetectable cTSH isoforms was not investigated in this study. The presence of concurrent NTI in dogs with hypothyroidism may have contributed to the finding of reference range cTSH results in a number of the dogs in the present study. However, it remains unclear why other dogs with hypothyroidism and concurrent NTI had the expected elevations in circulating cTSH. The severity and duration of illness may be of importance and is worthy of further study.

Based on the results of this study the measurement of cTSH in isolation cannot be recommended. However, of the cases with discordant cTSH results, only one of the hypothyroid dogs had a normal circulating total or free T_4 concentration whereas most euthyroid dogs had normal total T_4 and free T_4 concentrations. The combined estimation of either total or free T_4 in association with cTSH is therefore useful irrespective of disease status. It is clear from these results that concurrent estimation of additional thyroid parameters reduces the misinterpretation of discordant cTSH concentrations in the vast majority of cases. In the case of free T_4 in particular it is clear that very few animals are misdiagnosed as hypothyroid, when this is measured alongside cTSH supporting the results of similar studies (Peterson *et al.,* 1997). The diagnostic accuracy of cTSH was also dramatically improved when evaluated as part of a ratio with either total or free T_4. Overall, the calculation of the circulating free T_4 to cTSH ratio, was a better performing test as assessed by the area under the ROC curve, than the total T_4 to cTSH ratio. However, when used at their optimal cut-off values, the diagnostic performance of each method was similar. The use of total T_4 as a ratio was less sensitive and more specific than the free T_4 to cTSH ratio. This is in contrast to the performance of the analytes when evaluated alone. However, this was simply a consequence of the selected cut-off value, and it is clear from Figure 10 that the total T_4 to cTSH ratio DPR plateau is, as was the case with total T_4 alone, relatively flat across a range of cut-off values. The selection of a higher cut-off could therefore be adopted for the total T_4 to cTSH ratio if it was intended to be used primarily as a screening test.

The use of the bovine TSH response tests has been criticised because of the potential for anaphylactic reaction and the expense of the test (Nachreiner, 1991; Hasler & Rohner, 1992). In this study no adverse clinical signs were recognised in any of the dogs following the administration of TSH. The criteria used for performing and interpreting the test were similar to those most recently published (Frank, 1996; Peterson *et al.,* 1997; Scott-Moncrieff & Nelson, 1998). This protocol was practical for use in a clinical research setting and was not prohibitively expensive even in the largest dogs. Indeed the reliable

diagnosis of hypothyroidism with the TSH response test is more economically justifiable compared to the costs of inappropriate lifelong replacement therapy (Ferguson, 1991). It should be clear that whilst the bovine TSH response test is undoubtedly the most reliable means currently available for confirming canine hypothyroidism, it is not a perfect test and difficulties in case categorisation can still occur (Panciera, 1990b). Equivocal TSH response test results therefore remain a problem. However, the use of the equivocal category of TSH response test results, in association with case follow-up information and additional test results did allow confident classification of the dogs used in this study. This allows confidence in the conclusions reached in this study regarding the single-test analytes compared to some previous studies using less reliable methods for case categorisation (Ramsey *et al.,* 1997). The use of an equivocal category for the TSH response test was useful, and demonstrated that the majority of cases with borderline TSH response test results are ultimately confirmed as euthyroid.

In conclusion, the results of this study have identified a number of the limitations of the currently available tests of canine thyroid function and provide guidance on the most reliable approach to confirm hypothyroidism. Firstly, it is clear that numerous factors, but particularly recovery from NTI and sulphonamide therapy may influence test results. Therefore as far as is practical, the presence of NTI should be excluded and drug therapy be taken into account prior to performing thyroid function tests. If NTI cannot be completely excluded, dogs should be clinically stable when thyroid function tests are performed. Secondly, as an individual parameter, free T_4 estimation is the most reliable test for canine hypothyroidism followed by total T_4 measurement. However, given the higher cost of free versus total T_4 estimation, total T_4 is an economically justifiable solitary first test particularly if used primarily as a screening test. Thirdly, the use of circulating cTSH or total T_3 estimation alone cannot be recommended. However, it is clear that the use of two or more tests simultaneously, dramatically improves the accuracy for diagnosing hypothyroidism compared with single test analysis. In particular, the measurement of either total T_4 or free T_4 with cTSH provides a much more sensitive and specific assessment of thyroid status than any of the analytes individually. The use of total and free T_4 to cTSH ratios is also preferable to the individual analytes. However, because of the mathematical effects that use of a ratio can introduce, overall consideration of the multiple individual tests results remains of principal importance.

If only one tests is to be performed, free T_4 measurement is the most reliable method. If two tests are performed, either free or total T_4 should be measured with cTSH.

In cases with a discordant pair of results, an additional test usually clarifies the situation irrespective of thyroid status and rarely results in an erroneous diagnosis. The use of TgAb measurement is discussed in detail in Chapter 7 and although not specifically reported in this chapter, may be particularly beneficial in this regard. The greatest diagnostic dilemma probably arises in the dog investigated using the diagnostic protocol outlined above and in which decreased total T_4 and normal cTSH results are subsequently obtained. Measurement of any other analyte but particularly free T_4 concentration is advised in such a case. If after further testing, the diagnosis remain unclear, TSH response testing is the preferred option.

CHAPTER 5

EPIDEMIOLOGICAL, CLINICAL AND ROUTINE CLINICOPATHOLOGICAL FEATURES OF CANINE HYPOTHYROIDISM

5.1 LITERATURE REVIEW

Epidemiological Features of Hypothyroidism

Hypothyroidism is considered to be one of the most common endocrine disorders of dogs and several studies have previously evaluated some of the epidemiological features of the disease (Nesbitt, Izzo, Peterson & Wilkins, 1980; Milne & Hayes, 1981; Kaelin *et al.*, 1986; Panciera, 1994; Feldman & Nelson, 1996; Peterson *et al.*, 1997). However, these reports have been complicated by inconsistency in the diagnostic criteria applied for confirming hypothyroidism, particularly the use of what are now often considered unreliable diagnostic tests. Consequently, estimation of the prevalence of hypothyroidism varies between studies. Panciera, (1994) reported the prevalence to be 0.2 % using rigid criteria for confirmation of hypothyroidism, whereas Milne & Hayes, (1981) reported a prevalence of 0.64 % using much looser diagnostic criteria. Most large surveys of hypothyroidism have examined the canine population in the United States which is not necessarily representative of the UK dog population. This, in addition to the variable quality of diagnostic criteria used has resulted in a surprising lack of reliable epidemiological data regarding this disease worldwide, and in the UK in particular.

Age

Nesbitt *et al.*, (1980) evaluated 108 dogs tentatively diagnosed with hypothyroidism based on the demonstration of depressed circulating concentrations of total T_4 and/or total T_3. Clearly, these criteria are poorly specific for hypothyroidism and the results must be interpreted accordingly. The age of onset of clinical signs was recorded in 96 of the cases and was less than one year in 11 (11.5 %), between one and three years in 44 (45.8 %), between four and six years in 28 (29.2 %) and over seven years of age in 13 (13.5 %) dogs. Large breed dogs reportedly had an earlier age of onset compared to small breeds. However, these data were not provided.

Milne & Hayes, (1981) diagnosed hypothyroidism in 3184 cases out of 1.1 million dogs seen at multiple veterinary teaching hospitals. The diagnosis of hypothyroidism was confirmed using a variety of methods including the presence of appropriate clinical signs,

historical details, clinicopathological analysis and thyroid gland histopathological examination. One hundred and six (3.3 %) dogs were less than one year of age, 227 (7.1 %) were one to two years old, 741 (23.3 %) were between two and three years, 1058 (33.2 %) were between four and six years, 674 (21.2 %) were between seven and nine years and 378 (11.9 %) were greater than 10 years of age. The relative risk of age for the development of hypothyroidism was stratified by breed, using "high risk" and "low risk" breed categories. High risk breeds were reported to be more likely to develop hypothyroidism at an earlier age (up to three years of age) after which the risk slowly declined. This was interpreted by the authors as suggesting a genetic component in the aetiology of the disease. Conversely, low risk breeds tended to have a lower risk of hypothyroidism in the younger age categories, but this steadily increased until nine years of age.

Kaelin *et al.*, (1986) confirmed hypothyroidism with TSH response tests in 16 dogs. The protocol used for the test was based on the collection of blood samples immediately prior to and 12 hours following the subcutaneous or intramuscular administration of 5 i.u. or 10 i.u. bovine TSH to dogs less than or greater than 10 kg, respectively. The age of affected dogs was unknown in one case, and ranged from 0.3 to 11 years (median six years) in the remaining 15 animals. Thirteen of the dogs were at least four years of age and the authors reported that the two animals under this age (aged 0.3 and 0.8 years) were likely to have had congenital hypothyroidism.

Panciera, (1994) confirmed hypothyroidism in 66 dogs based on the results of TSH response tests. Mean age at the time of diagnosis was 7.2 years (range 0.5 to 15 years).

Median age in the 54 dogs with confirmed hypothyroidism reported by Peterson *et al.*, (1997), who also used bovine TSH response tests to classify cases, was six years (range 2 to 13; interquartile range 4 to 7 years) compared to a median of 10 years (range 2 to 17; interquartile range 6 to 13 years) in 54 euthyroid dogs. No statistical analyses of these results were reported or performed.

Breed

Nesbitt *et al.*, (1980) reported the breed distribution of 108 hypothyroid dogs which included 13 (12 %) doberman pinschers, eight (7.5 %) great Danes, seven (6.5 %) each of poodles, dachshunds, and schnauzers, and six (5.5 %) each of Irish setters and boxers. The remaining 54 (50 %) dogs were composed of 27 different breeds. Large and giant breeds accounted for approximately one third of the cases in the study. However, no information

was provided regarding the distribution of these and other breeds within the non-hypothyroid population examined.

Milne & Hayes, (1981) determined the effect of breed on the risk of developing hypothyroidism by calculating the relative risk with the associated 95 % confidence intervals for each breed. Airedale terriers, miniature and standard dachshunds, doberman pinschers, golden retrievers, miniature schnauzers, Irish setters, Shetland sheepdogs, cocker spaniels and pomeranian dogs were found to be at significantly increased risk of hypothyroidism. German shepherds and mixed breed dogs were significantly less likely to develop hypothyroidism than other breeds. The assessment of relative risk was made by comparison of the relative number of each breed in which a diagnosis of hypothyroidism was made, compared to the number of that breed with diseases other than hypothyroidism. The population studied consisted of animals seen at 15 veterinary teaching hospitals and it is therefore likely that these data represented mainly second-opinion cases.

Statistical analysis of the breeds of hypothyroid dogs reported by Kaelin *et al.,* (1986) was not possible due to the small number of cases. The 16 cases included two doberman pinschers, one cocker spaniel, one schnauzer, nine dogs of various other breeds and three crossbreed dogs.

Panciera, (1994) determined the effect of breed on the likelihood of developing hypothyroidism using chi squared analysis and calculated odds ratios (o.r.) for each category. The reference population was considered to be all dogs examined at the same hospital during the study period in which a diagnosis of hypothyroidism was not made. Of the 66 hypothyroid dogs, doberman pinschers (n=10, o.r.=6.06) and golden retrievers (n=11, o.r.=2.86) were significantly at risk of developing hypothyroidism compared with other breeds. Crossbreed dogs accounted for nine cases, and no other breed was represented by more than three individuals.

Feldman & Nelson, (1996) diagnosed hypothyroidism in 130 cases using unspecified criteria and reported the breeds most commonly affected to be golden retrievers (n=24, 18 %), doberman pinschers (n=22, 17 %), Labrador retrievers (n=8, 6 %), cocker spaniels (n=7, 5 %), German shepherds (n=7, 5 %) and crossbreed (n=7, 5 %) dogs.

Peterson *et al.,* (1997) reported the most commonly affected breeds to be golden retrievers (n=8, 15 %), cocker spaniels (n=6, 11 %), doberman pinschers (n=5, 9 %), dalmations (n=4, 7 %), and two each of boxers, German shepherds, Labrador retrievers, poodles and Shetland sheepdogs. Six (11 %) dogs were crossbreeds and the remaining 15 dogs represented 15 other breeds. However, the same study reported a similar distribution

of breeds within the euthyroid group suggesting that either certain breeds were more prevalent in the referral population studied, or were more frequently tested for hypothyroidism.

Clearly, several breeds including the doberman pinscher, golden retriever and cocker spaniel have been reported to be particularly prone to hypothyroidism. The view is also widely held that purebred dogs are more frequently affected than crossbreeds. This has been presumed to indicate a genetic predisposition to the development of hypothyroidism (Milne & Hayes, 1981). The increased incidence of thyroiditis and thyroglobulin autoantibodies within particular breeds and families of dog has supported this contention (Conaway *et al.,* 1985; Benjamin, Stephens, Hamilton, Saunders, Lee, Angleton & Mallinckrodt, 1996) and is discussed in detail in Chapter 7. However, much of the published data concerning the prevalence of thyroiditis has been performed with experimental animals, particularly beagles, a breed known to be prone to the development of thyroiditis (Benjamin *et al.,* 1996). The role that pedigree status and breed have on the incidence of clinical hypothyroidism cannot necessarily be inferred from these experimental studies.

Gender

Nesbitt *et al.,* (1980) reported a roughly equal number of hypothyroid males (n=55) and females (n=53). However, no data were provided regarding the neutering status of these animals.

Of the 3184 hypothyroid dogs reported by Milne & Hayes, (1981), 1811 (56.9 %) were female (733 neutered) and 1373 (43.1 %) were male (89 neutered). After controlling for the effect of age and breed, there was no difference in the risk of developing hypothyroidism between sexually intact males and females. Male castrated dogs were at 30 % increased risk compared to intact males but this was not statistically significant. However, neutered female dogs were significantly at risk compared to intact females. This effect was particularly marked in the high risk breeds.

Kaelin *et al.,* (1986) reported hypothyroidism in 16 dogs of which six were female (four neutered) and 10 male (one neutered). No statistical analysis of these data were performed.

Panciera, (1994) reported that 30 of 66 (45 %) hypothyroid dogs were male (18 neutered) compared to 36 (55 %) females (30 neutered). Neutered males (o.r.=4.18) and neutered females (o.r.=3.46) were significantly at risk of developing hypothyroidism

compared with intact females. Neutered dogs regardless of sex were reported to be significantly at risk of developing hypothyroidism compared to intact animals. The influence of neutering was apparent both before and after stratification of the data by age.

Thirty one of 54 (57 %) hypothyroid dogs reported by Peterson *et al.*, (1997) were female (22 neutered) compared to 23 male (12 neutered). The euthyroid group in the same study consisted of 26 (48 %) female (20 neutered) and 28 (52 %) male (15 neutered) dogs. Although not specifically reported, chi squared analysis of these data revealed no significant difference in the sex or neutering status between the hypothyroid and euthyroid groups.

It is known that sex hormones have a mild effect on thyroid function in the dog and the relevant studies have been reviewed in Chapter 4. It has been suggested that the association between neutering and hypothyroidism may in fact be related to the influence of sex hormones on the immune system. In experimental mice, castration increases the severity of autoimmune thyroiditis (Okayasu, Kong & Rose, 1981). However, administration of oestrogens to ovariectomised female and castrated male rats ameliorates pre-existing thyroiditis. By contrast, progesterone administration to these individuals worsens the condition (Ahmed, Young & Penhale, 1983). The influence of sex and neutering status on the presence of TgAb in dogs is discussed in Chapter 7.

In humans, a number of variables including iodine consumption, gender and pregnancy play a role in the susceptibility to thyroiditis (Weetman, 1996). There is also an inverse correlation between birth weight and the prevalence of TgAb in women (Phillips, Cooper, Fall, Prentice, Osmond, Barker & Rees Smith, 1993). Clearly several mechanisms are likely to play a role in the development of this disease in all species, and further studies will be required to clarify these in dogs.

Clinical Features of Hypothyroidism

Thyroid hormones play a role in numerous cellular metabolic processes, discussed in detail in Chapter 4. Therefore deficiency of thyroid hormones can have diverse biological effects. There is widespread agreement that the clinical features of canine hypothyroidism are variable and non-specific (Feldman & Nelson, 1996). In addition the insidious nature of hypothyroidism makes appreciation of the clinical changes more difficult.

Dermatological Features

Thyroid hormones play several important roles in the maintenance of dermal health and hypothyroidism is the most common endocrine disease affecting the skin (Scott, 1982; Feldman & Nelson, 1996). Dermatological abnormalities in hypothyroidism can be extensive and are reported in the majority of affected dogs (Panciera, 1994). Thyroid hormones are necessary for the initiation of the anagen phase of the hair follicle cycle. Absence of thyroid hormones results in persistence of the telogen growth phase, hairs become easily epilated and eventually alopecia results. This commonly starts in areas undergoing friction such as the neck in dogs that wear collars and on the tail resulting in the "rat tail" typical of hypothyroidism. The alopecia is usually bilaterally symmetric in appearance but focal, mulitfocal and asymmetric alopecia can occur. Hair loss slowly progresses and will ultimately affect the flanks and trunk. The extremities are usually spared although giant breeds may suffer from alopecia of the extremities whilst the trunk remains relatively unaffected (Panciera, 1997b). The hair that remains is usually dry and brittle and may become lighter in colour presumably due to environmental bleaching. Some variation in lesion type and distribution may be breed related with Artic breeds often losing primary hairs giving a woolly coat appearance, daschunds developing pinnal alopecia, nasal alopecia occurring particularly in retrievers, and hypertrichosis being reported in boxers and Irish setters (Rosychuk, 1997). Hyperkeratosis occurs causing scaling and scurfing of the skin (Panciera, 1990a). Accumulation of dermal mucopolysaccharides and hyaluronic acid occurs due to an imbalance in the normal thyroid hormone-controlled production and degradation of these molecules, and results in myxoedematous skin thickening which is a common complaint in affected humans (Bernhard, Freedberg & Vogel, 1996). Dog owners rarely notice such a change in their pets, although the skin thickening can give rise to the so-called "tragic facial expression" due to thickening of the lips, over the forehead and drooping of the eyelids. Thyroid hormones assist the humoral and cellular immune responses, and consequently hypothyroidism reduces resistance to infection. Secondary recurrent and persistent superficial and deep pyodermas are therefore commonly reported in hypothyroidism (Peterson & Ferguson, 1989; Panciera, 1990a; Rosychuk, 1997). These infections may be poorly responsive to appropriate antibiotic therapy until the hypothyroidism is controlled. Inflammatory lesions often include accumulation of lymphocytes, neutrophils, macrophages and plasma cells and are presumably related to either mild secondary pyoderma and/or the irritation associated with seborrhoeic changes. Such abnormalities may result in pruritis with secondary self-induced excoriation and

trauma which can complicate the clinical and histopathological analysis. Other dermatohistopathological changes supportive of hypothyroidism include dermal thickening reported in 41 %, myxoedema reported in approximately 30 % and vacuolation of the arrector pili muscles reported in as many as 75 % of cases (Scott, 1982; Yeager & Wilcock, 1994).

In a study of 108 hypothyroid dogs, Nesbitt *et al.,* (1980) reported alopecia in 92 (85 %), loss of coat condition including dryness, dullness, dandruff, scaling and hair coarseness in 51 (47 %), hyperpigmentation in 34 (31 %) and pyoderma in 30 (28 %). Kaelin *et al.,* (1986) documented alopecia in 12 of 16 (75 %) hypothyroid dogs. Skin biopsies in five of these cases revealed hyperkeratosis and follicular plugging, and in four of them there was hyperpigmentation. In two dogs the dermis was thickened. In two cases, the arrector pili muscles were hypertrophic and vacuolation was also evident in one. These latter changes are considered to be particularly characteristic of hypothyroidism (Scott, 1982). Panciera, (1994) reported dermatological abnormalities in 39 of 66 (59 %) hypothyroid dogs, most commonly seborrhoea (n=26, 39 %), alopecia (n=17, 26 %) and pyoderma (n=7, 11 %). The alopecia was frequently bilaterally symmetric although hair loss in areas undergoing friction was also common.

Metabolic Features

A range of clinical signs associated with hypothyroidism including weight gain, lethargy, exercise intolerance and weakness are collectively described as "metabolic" abnormalities. These signs are considered to be manifestations of the reduced metabolic rate which occurs in these individuals (Panciera, 1997b). In hypothyroid humans, basal metabolic rate reduces by up to 40 % and the rate of heat production falls, reflecting the reduction in a wide variety of energy-generating reactions. This is often associated with cold intolerance and some degree of weight gain despite a decrease in appetite. The onset of metabolic signs is typically insidious often making their appreciation by owners particularly difficult. However, the clinical response following appropriate therapy can be dramatic, retrospectively confirming the extent of the problem. Anecdotal comments in the literature suggest that the metabolic signs associated with central hypothyroidism are less severe than primary hypothyroidism (Panciera, 1990a; Rijnberk, 1996). However, no evidence for this assertion has been published.

Nesbitt *et al.,* (1980) reported lethargy and obesity in 12 (11 %) and nine (8 %) of 108 hypothyroid dogs, respectively. Eleven of 16 (69 %) hypothyroid dogs reported by

Kaelin *et al.,* (1986) were obese and the same number were lethargic. Two cases (12 %) were reported to be hypothermic. Panciera, (1994) reported obesity to be common but generally mild although several individuals were grossly obese, weighing over 75 % more than the ideal breed weight. Lethargy was recorded in 13 (20 %) of the 66 cases. Feldman & Nelson, (1996) reported weight gain (n=48, 48 %), lethargy and mental dullness (n=35, 35 %) to be the most prevalent clinical abnormalities in a study of 100 dogs with hypothyroidism. That study suggested that the severity of metabolic signs are related to the stage of the thyroid disease which may account for the differences reported between studies.

Neuromuscular Features

Neuromuscular abnormalities including generalised lower motor neurone (LMN) paresis, peripheral vestibular disease, megaoesophagus, laryngeal paralysis, primary encephalopathy, cranial nerve disorders, mononeuropathies, neuromuscular junction disorders, myopathies and seizures have been variously reported in hypothyroid dogs (Braund, Dillon, August & Ganjam, 1981; Chastain, Graham & Riley, 1982; Dewey, Shelton, Bailey, Willard, Podell & Collins, 1995; Coates, 1997). Neurological signs were recorded in 19 of 66 (29 %) hypothyroid dogs reported by Panciera, (1994) although some of these individuals had more than one neurological complaint. Four dogs had unilateral and one had bilateral facial nerve paralysis. Four dogs had unilateral peripheral vestibular nerve disease of which three dogs had additional neurological deficits. Three dogs had generalised neuromuscular disease. Six dogs (all of which were doberman pinschers) had cervical spondylomyelopathy, five had laryngeal paralysis and five had megaoesophagus. Jaggy, Oliver, Ferguson, Mahaffey and Glaus Jun, (1994) reported lower motor neuron signs in 11 (38 %), peripheral vestibular deficits in nine (31 %), laryngeal paralysis in five (17 %) and megaoesophagus in four (14 %) of 29 hypothyroid dogs with neurological signs. In each case the diagnosis had been based on TSH response tests and a positive response to thyroid hormone medication with a follow up period of at least six months. Many of the dogs with other neurological signs had concurrent generalised LMN signs which was considered the most common clinical manifestation. However, some studies of canine hypothyroidism including 16 dogs reported by Kaelin *et al.,* (1986) and 108 dogs reported by Nesbitt *et al.,* (1980) make no reference to neuromuscular abnormalities.

The pathological basis for hypothyroid neuropathies is most likely due to axonal degeneration and demyelination (Pollard, 1993). However, confirmation of a genuine

cause-effect relationship of certain neuromuscular abnormalities, particularly megaoesophagus and laryngeal paralysis is complicated by the variable response of these conditions to thyroid hormone supplementation and their regular occurrence in euthyroid dogs (Jones, Jergens & Guilford, 1989; Jaggy & Oliver, 1994; Jaggy *et al.,* 1994). The true role of thyroid dysfunction in their development therefore remains to be clarified. Variation in the quality of case detail recording and classification of cases may also partly account for the differences reported between studies.

Neuromuscular signs are relatively common in human patients with hypothyroidism. These include peripheral neuropathies in up to 75 % of patients, and less frequently central nervous system abnormalities (DeLong, 1996). The pathological basis for these changes appears to vary but may include axonal degeneration, slowed conduction velocities or subcellular cerebellar deficits. Appropriate thyroid hormone replacement therapy in affected human patients is usually curative although recovery can take up to two years in severely affected individuals. It is currently unclear if the higher incidence of neurological symptoms reported in humans compared to hypothyroid dogs is related to a genuine species difference or a reduced detection of the more subtle neurological signs in dogs.

Cardiovascular Features

Thyroid hormones act on the myocardium by stimulating myocardial hypertrophy, exerting a positive inotropic effect and increasing responsiveness to adrenergic stimulation (Kienle, 1997). In humans, thyroid hormones regulate these mechanisms by varying the expression of a number of myocyte contractile proteins and altering both myocyte sodium and calcium transport and mitochondrial function. Consequently, thyroid hormones can rapidly increase cardiac contractility and lower systemic vascular resistance. Correspondingly, in human hypothyroid patients, all measurements of left ventricular performance including cardiac output, stroke volume and heart rate are significantly decreased. In addition the rate of diastolic ventricular relaxation reduces, and wall compliance diminishes further impairing cardiac performance (Klein & Ojamaa, 1996).

In dogs the effect of thyroid hormones on cardiac function is much less clearly defined. The most frequently recognised electrocardiographic abnormalities in hypothyroid dogs are small QRS complexes, inverted T waves and sinus bradycardia (Nijhuis, Stokhuff, Huisman & Rijnberk, 1978). There appears to be a direct correlation of the severity of the electrocardiographic (ECG) abnormalities with the degree of hypothyroidism (Nijhuis *et al.,* 1978). These changes typically reverse with appropriate hormone replacement therapy.

Experimentally induced hypothyroidism has also been demonstrated to reduce myocardial function in dogs (Santos, Miller & Mathew, 1980). However, spontaneous congestive cardiac failure appears a rare complication of hypothyroidism unless in the presence of concurrent cardiac disease.

The possibility of a relationship between hypothyroidism and cardiac disease, specifically dilated cardiomyopathy (DCM) has been frequently suggested in the literature. However, there is surprisingly little solid evidence for a genuine cause and effect relationship between the two conditions. Calvert, Chapman & Toal, (1982) evaluated thyroid status in 13 dogs with spontaneous DCM. Only one of these dogs had an abnormal response to exogenous TSH administration consistent with hypothyroidism. Panciera & Refsal, (1994) evaluated the effect of spontaneous cardiac failure on thyroid function and reported no significant difference between those animals with clinical heart disease, and healthy control animals. More recently Calvert, Jacobs, Medleau, Pickus, Brown & McDermott, (1998) evaluated the results of bovine TSH response tests in 152 doberman pinschers with various diseases. Cardiomyopathy was confirmed in 79 (52 %) cases of which 23 (29 %) were classified as hypothyroid. However, 29 of the 73 (40 %) non-DCM cases were also reported to be hypothyroid and there was no significant difference in the prevalence of cardiac disease between the hypothyroid and euthyroid dogs. Furthermore, of those dogs with DCM, the thyroid status was not significantly different between the sub-groups with or without congestive cardiac failure. This large and well controlled study supports the comments by Kienle (1997) that a relationship between hypothyroidism and clinical cardiac failure remains to be proven.

Reproductive Features

Clinical reproductive abnormalities including persistent anoestrus, galactorrhoea, infertility and prolonged inter-oestrus interval in the bitch, and decreased fertility and libido in the male dog have all been attributed to hypothyroidism (Chastain & Schmidt, 1980; Johnson, Larsen & Olsen, 1982; Feldman & Nelson, 1996). However, the mechanism of the interrelationship between thyroid hormones and reproductive function remains unclear and whilst there is evidence of an association between lymphocytic thyroiditis and lymphocytic orchitis in genetically susceptible beagles (Fritz, Lombard, Tyler & Norris, 1976) the data confirming spontaneous hypothyroidism as the cause of clinical reproductive abnormalities is in fact sparse. Nesbitt *et al.*, (1980) documented abnormal oestrus in five of 108 (5 %) dogs with spontaneous hypothyroidism. However, more specific details of the reproductive

problems were not provided. Panciera, (1994) reported reproductive abnormalities (one each of decreased libido and a history of abortion) in two of 66 (3 %) hypothyroid dogs classified by bovine TSH response tests. Johnson, Olivier, Nachreiner & Mullaney, (1999) reported no decrease in male reproductive performance as assessed by daily sperm production, ability to ejaculate and total scrotal width in six beagles two years following radiothyroidectomy. Clinical reproductive disease as a result of hypothyroidism therefore appears to be a relatively uncommon feature.

Other Features

A variety of other clinical signs have been less frequently associated with canine hypothyroidism. Corneal lipidosis associated with hypothyroidism and hyperlipidaemia has previously only been described in German shepherd dogs (Crispin & Barnett, 1978). Gastrointestinal disease, particularly constipation related to impaired motility, has been reported in humans and if left untreated this can progress to a state of pseudo-obstruction. There is similar data relating to affected dogs (Feldman & Nelson, 1996). More recently diarrhoea resulting from small intestinal bacterial overgrowth (SIBO) has also been reported as a gastrointestinal feature of canine hypothyroidism (Jackson, Jackson, Wotton & Hall, 1998). The authors had no evidence for an underlying mechanism to link the two conditions, but considered the possibility of reduced intestinal motility, predisposing to bacterial overgrowth.

Clinicopathological Features of Hypothyroidism

There are well described biochemical and haematological abnormalities associated with hypothyroidism in both humans and dogs. However, these changes are usually compared with laboratory reference ranges, and are widely accepted as being neither sensitive nor specific for the condition. Consequently routine clinicopathological changes are considered merely supportive of a clinical diagnosis and the results of additional endocrine testing. The most common abnormalities reported in the literature include anaemia, hyperlipidaemia, and elevated circulating creatine kinase (CK) concentration.

Anaemia

Haematological changes associated with hypothyroidism have been recognised in humans since the late 1800s. Similar changes are now also reported in affected dogs. The anaemia, often termed a "physiological" anaemia is most commonly normocytic normochromic, and is presumed to be related to the reduction in metabolic and therefore oxygen requirements in affected patients. Various other types of anaemia have also been reported in human hypothyroid patients including macrocytic, and hypochromic microcytic anaemias. These abnormalities result from a variety of mechanisms including decreased erythropoietin production associated with reduced oxygen demand, reduced bone marrow activity, haemolysis, iron deficiency and idiopathic forms (Green & Ng, 1986). A reduction in peripheral tissue oxygen utilisation and impaired gastrointestinal iron absorption are also likely to be partly responsible for the red blood cell changes found in thyroid deficient states. The severity and chronicity of hypothyroidism is generally reflected by the degree of anaemia. However, this is not always the case and the changes are not specific for hypothyroidism.

Kaelin *et al.,* (1986) reported a normocytic normochromic anaemia in five of 12 (42 %) hypothyroid dogs classified based on inadequate response to TSH administration. Panciera, (1994) reported the same abnormality in 18 of 57 (32 %) hypothyroid dogs which were also confidently diagnosed. Feldman & Nelson, (1996) reported anaemia in under 50 percent of their dogs but more detailed data were not provided.

Hyperlipidaemia

Alterations in lipid metabolism are associated with hypothyroidism and result from changes in a number metabolic processes. Abnormalities of the triglyceride rich lipoproteins (chylomicrons and very low density lipoproteins (VLDL)), and cholesterol-rich lipoproteins (low density lipoproteins (LDL) and high density liporpoteins (HDL)) may all play a role in the alteration of the lipid profile in affected animals. In dogs as well as humans, this "hypothyroid hyperlipidaemia" is primarily caused by a reduction in the rate of lipid degradation (Peterson & Ferguson, 1989). Thyroid hormones enhance the degradation of cholesterol to bile acids and may increase the sensitivity of adipose tissue to the lipolytic effects of several hormones. In addition, the activity of lipoprotein lipase is diminished in thyroid hormone deficient states (Zerbe, 1986). Whilst there is a concomitant reduction in lipid synthesis in hypothyroidism the degradation is affected to a greater extent and the net effect is therefore an accumulation within the circulation (Feldman & Nelson, 1996).

Kaelin *et al.,* (1986) reported an increased blood cholesterol in 10 of 14 (71 %) hypothyroid dogs which is similar to 40 of 55 (73 %) hypothyroid cases reported by Panciera, (1994). Peterson & Ferguson, (1989) reviewed the literature of hypothyroid dogs categorised by TSH response tests and reported hypercholesterolaemia in 45 of 56 (80 %) cases. Feldman & Nelson, (1996) report hypercholesterolaemia in over 75 % of their hypothyroid dogs. However, more detailed data were not provided. The magnitude of hypercholesterolaemia tends to be greater in hypothyroidism than in other illnesses including other endocrine diseases (Barrie, Watson, Stear & Nash, 1993). Therefore whilst hypercholesterolaemia is not specific for hypothyroidism, an unusually exaggerated increase in cholesterol concentration should prompt consideration of hypothyroidism in particular.

Data on the severity and frequency of abnormalities in triglyceride concentrations in hypothyroidism are sparse. However, anecdotally, the changes in circulating triglyceride concentrations are less consistent than for cholesterol (Peterson & Ferguson, 1989; Zerbe, 1986). Rogers, Donovan & Kociba, (1975) diagnosed hypothyroidism in 26 dogs and reported increased concentrations of serum triglycerides, cholesterol and occasionally free glycerol. In cases with less severe hyperlipidaemia, only hypercholesterolaemia occurred. However, the diagnostic criteria used for confirming hypothyroidism in this study were unreliable with a number not even having circulating total T_4 estimations performed.

Creatine Kinase

Elevations in CK have been previously reported in hypothyroidism. However, the aetiology of this abnormality is unclear and whilst it is commonly assumed to result from a hypothyroid myopathy, hypothyroid dogs with histologically confirmed myopathy can have normal CK results whilst others without abnormal muscle biopsy findings may have elevated CK concentrations (Braund *et al.,* 1981). Karlsberg & Roberts, (1978) investigated the pharmacokinetics of circulating CK in four healthy dogs prior to and following the induction of hypothyroidism by ^{131}I radiothyroidectomy. That study suggested that the increased CK concentration may in fact be related to a decreased rate of plasma clearance.

Panciera, (1994) reported elevated CK in nine of 51 (18 %) hypothyroid dogs although the increase tended to be marginal. Jaggy *et al.,* (1994) reported a similar finding in three of 29 (10 %) hypothyroid dogs with neurological signs.

Other clinicopathological abnormalities

Several additional clinicopathological abnormalities have been reported in hypothyroidism including mild hypercalcaemia, increased alkaline phosphatase activity, thrombocytosis and decreased mean platelet volume (Kaneko, 1989; Sullivan, Gompf, Schmeitzel, Clift, Cottrell & McDonald, 1993; Panciera, 1994). However, none of these abnormalities have been considered to be of diagnostic utility due to their insensitive and non-specific pattern of change in hypothyroid dogs and those with NTI.

5.2. INTRODUCTION

There are widely held preconceptions regarding the typical epidemiological, clinical and clinicopathological features of hypothyroidism in the dog. However, surprisingly few published reports of these characteristics have been based on cases in which hypothyroidism has been confirmed using appropriate and accurate diagnostic criteria. In addition, most of the studies reported elsewhere have examined dogs from the United States or Australia (Nesbitt *et al.,* 1980; Milne & Hayes, 1981; Kaelin *et al.,* 1986; Panciera, 1994). These data may not therefore be truly representative of the UK dog population. The clinical and clinicopathological features of hypothyroidism have been previously reported (Panciera, 1994). However, like most other similar studies, the clinicopathological abnormalities in affected dogs were compared with laboratory reference ranges. Consequently the specificity of these features for hypothyroidism remains unclear since the incidence of similar abnormalities in dogs in which hypothyroidism is suspected but excluded, has not been reported.

This study was designed to evaluate the epidemiological, clinical and clinicopathological features of confidently diagnosed hypothyroidism in a population of dogs from the UK. A novel approach was to determine the diagnostic value of routine biochemical and haematological parameters by identification of those analytes which most reliably distinguished hypothyroid dogs from those with clinically similar illness but in which a diagnosis of hypothyroidism was ultimately excluded.

5.3 MATERIALS AND METHODS

Case Material and Classification

The case material comprised a series of 140 dogs referred to the University of Glasgow Veterinary School and subsequently investigated for suspected hypothyroidism as outlined in Chapter 2. Based on the total T_4 response to exogenous stimulation with bovine TSH, the dogs were classified as either hypothyroid (n=52) or euthyroid (n=88) using the criteria detailed in Chapter 4. Results of hormone analyses from these cases are detailed in Chapter 4.

Routine Clinicopathological Analysis

Routine biochemical and haematological analyses were performed as described in Chapter 2.

Clinical Control Groups

Three groups of cases categorised as the euthyroid, hospital and external groups were used as the clinical control groups as outlined in Chapter 2. To assess if the distribution of breeds within the referral group were likely to be influenced by particular specialities or disciplines within DVCS, the hospital system was interrogated as outlined in Chapter 2. The referral group was subdivided according to the speciality to which the cases were referred, for example, orthopaedics, ophthalmology, neurology, general medicine etc. The cases within each discipline were then subdivided by breed. The intention of this was to allow comparison of breed distribution between disciplines and identify any specialities in which there was marked over-referral of a particular breed. It was conceded that this approach would not allow the identification of disciplines in which breeds were significantly underrepresented. Only disciplines with greater than 10 cases of a single breed constituting at least 10 percent of the total number of referrals of that breed were analysed. These values were chosen arbitrarily but it was considered that any biases within a discipline which involved under 10 percent of the total referrals of that breed would be unlikely to have a marked effect on the general control population. It was intended that any subsets with a confirmed bias would be removed prior to statistical analyses.

Clinicopathological Control Groups

Results of biochemical and haematological analyses from the hypothyroid dogs were compared with clinicopathological controls as outlined in Chapter 2.

Data Manipulation

Clinicopathological results were recorded, stored and analysed as outlined in Chapter 2. All results are reported as median (semi-interquartile range (SIR)). The association of sex, neutering status and pedigree status with the development of hypothyroidism was determined by calculating o.r.'s with the associated 95 % confidence intervals using each of the two clinical control groups. The ages of the hypothyroid, euthyroid and hospital groups were compared using analysis of variance techniques with post hoc pairwise comparison of means using Newman-Keuls multiple range tests. The association of breed with hypothyroidism was determined by chi-squared analysis using each of the clinical control groups and breeds represented by at least five cases in each group. A Mann-Whitney U test was used to compare routine haematological and biochemical results between the hypothyroid and euthyroid dogs. Diagnostic sensitivity and specificity were defined as

described in Chapter 2. Diagnostic efficiency of individual analytes was defined as the percentage of results which correctly classified cases irrespective of their disease status.

5.4 RESULTS

Signalment

The hypothyroid group consisted of 23 male (5 neutered) and 29 female (15 neutered) animals ranging in age from three to 13 years (mean ± s.d., 7.6 ± 2.6 years). Of these animals 46 were classified as pedigree and the remaining six as crossbreeds. The breed types and breeds most commonly affected are detailed in Table 5 and displayed in Figure 14. The euthyroid group consisted of 49 male (10 neutered) and 39 female (22 neutered) animals ranging in age from one to 14.5 years (mean ± s.d., 7.3 ± 3.3 years). Of these animals 83 were classified as pedigree and the remaining five as crossbreeds. The breed types and breeds are detailed in Table 5 and displayed in Figure 15. The relevant hospital group during the study period consisted of 3040 male (588 neutered) and 2722 female (1371 neutered) dogs ranging in age from 0.02 to 22.8 years (mean ± s.d., 5.9 ± 3.8 years). Of these animals 5315 were classified as pedigree and the remaining 447 as crossbreeds. The breed types and breeds most commonly referred are detailed in Table 5 and displayed in Figure 16. The distribution of breeds within each referral discipline, and the attempted analysis of these data are detailed in Appendix 31. The external group consisted of a total of 1,052629 dogs distributed between 192 breeds. These cases are detailed in Table 5.

The most common breeds and breed types in the study are displayed in Figure 17. There was no significant difference in breed distribution between the hypothyroid and either the euthyroid, hospital or external control groups. However, there were significant differences between the euthyroid and external ($p<0.001$) groups with collies being markedly over-represented in the euthyroid group. There was also a significant ($p<0.001$) difference between the hospital and external group breeds, with collies, terriers, rottweilers and doberman pinschers being most over-represented in the hospital group. Despite this, eight of the 10 numerically largest breeds in the external group were amongst the 10 most frequently represented breeds in the hospital group.

The pedigree distribution of each group is shown in Figure 18. There was no significant difference in pedigree status between the hypothyroid and both the euthyroid and hospital groups. No comparison with the external group could be made.

Breed	Hypothyroid	Euthyroid	Hospital	External
Retrievers	10	22	1015	199098
Spaniels	7	6	632	114927
Terriers	6	12	941	151992
Crossbreeds	6	5	447	0
Doberman	3	4	96	10014
Japanese Akita	2	0	21	4302
Collies	2	8	489	20971
Schnauzer	2	1	37	10594
Sheltie	2	2	49	11866
Rottweiler	2	3	143	15376
Boxer	1	3	178	38586
German shepherd	1	1	484	96485
Griffon	1	0	4	500
Hovawart	1	0	0	78
Pointers	1	0	37	9251
Poodle	1	0	99	19456
Pug	1	0	5	2144
St Bernard	1	0	32	3296
Setters	1	4	79	12268
Viszla	1	0	16	1800
Beagle	0	0	27	3809
Belgian Shepherd	0	1	3	1950
Bernese Mountain Dog	0	1	40	3133
Bichon Friese	0	1	18	10778
Borzoi	0	1	1	745
Bullmastiff	0	1	56	7847
Chow chow	0	2	28	2830
Dachshund	0	2	50	18424
Dalmation	0	1	28	13610
Lhasa Apso	0	2	31	12742
Newfoundland	0	1	43	3758
Pekinese	0	1	27	6264
Pyrenean Mountain Dog	0	1	12	1221
Samoyed	0	1	31	4849
Shih Tzu	0	1	31	17973
Others	0	0	532	219692
Totals	**52**	**88**	**5762**	**1052629**

Table 5. Distribution of the breed-types of the hypothyroid, euthyroid, hospital and external groups of dogs.

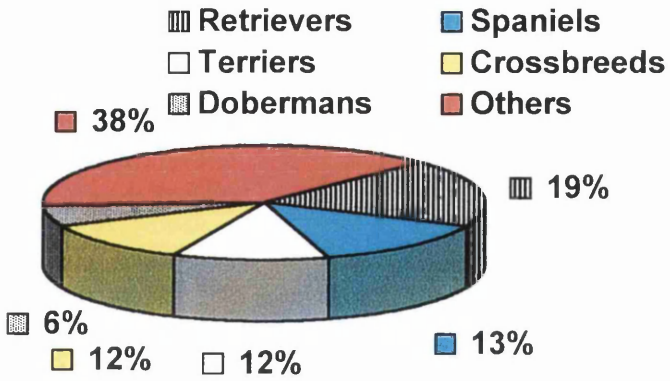


Figure 14. Distribution of breed-type of 52 dogs with confirmed hypothyroidism.

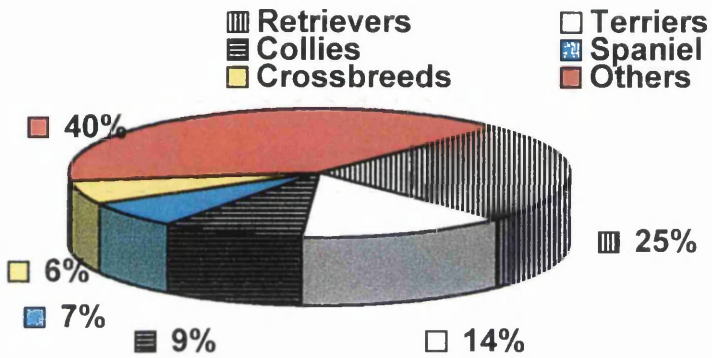


Figure 15. Distribution of breed-type of 88 euthyroid dogs investigated for hypothyroidism but in which alternative diagnoses were made.

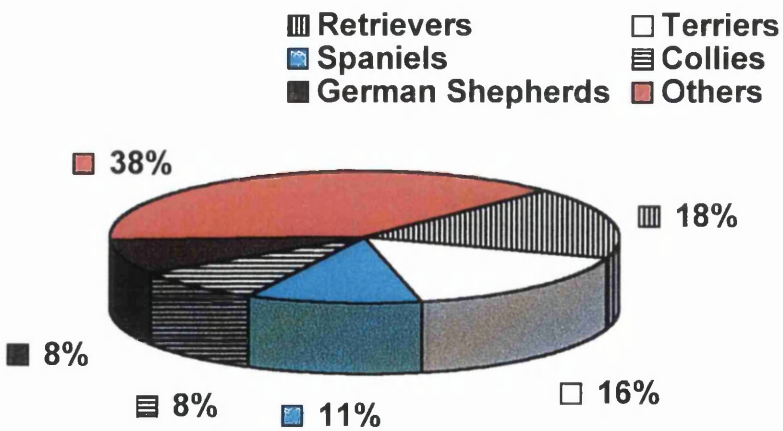


Figure 16. Distribution of breed-type of all referred dogs which were not investigated for hypothyroidism.

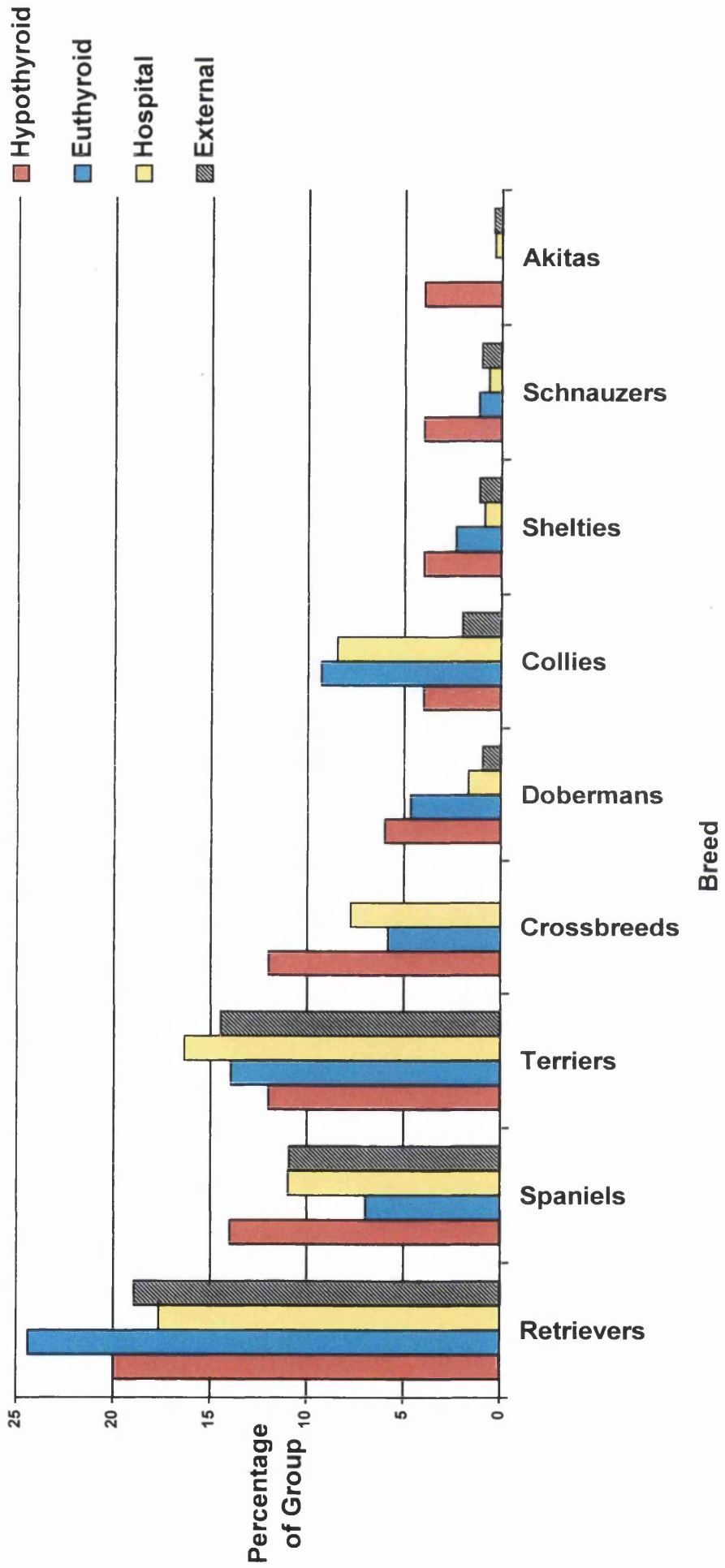


Figure 17. Distribution of the most commonly represented breeds from the hypothyroid, euthyroid, hospital and external groups of dogs.

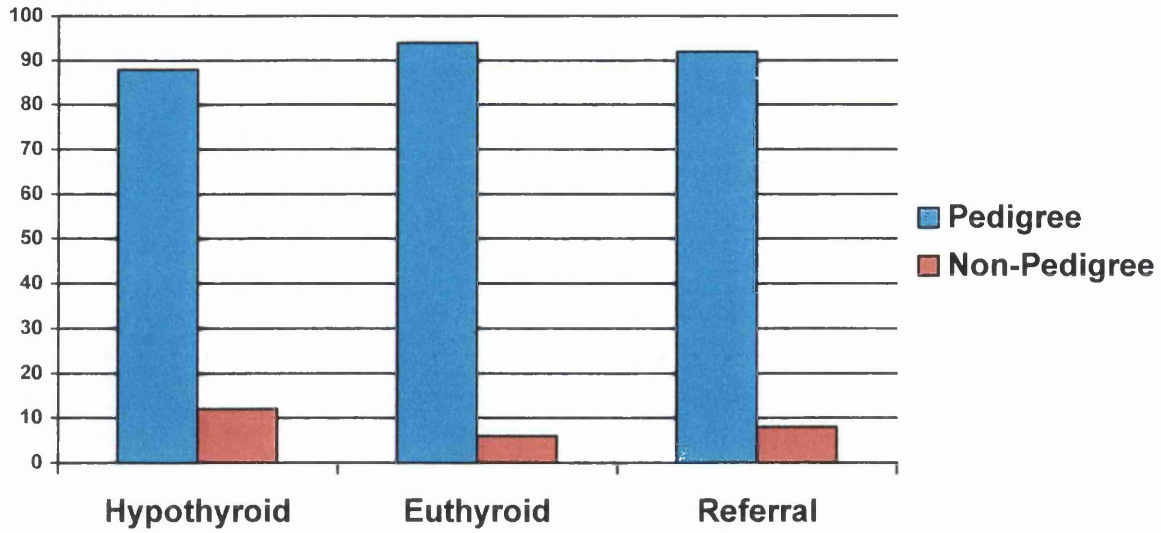


Figure 18. Relative numbers of the hypothyroid, euthyroid and referral groups of dogs which classified as either pedigree or non-pedigree.

Details of the sex and neutering distribution of dogs in the hypothyroid, euthyroid and hospital groups are shown in Table 6. There was no significant difference in sex or neutering status between the hypothyroid and either the euthyroid or hospital control groups. No comparison with the external group could be made.

Group	Hypothyroid	Euthyroid	Hospital
Male Entire	18	39	2452
Male Neutered	5	10	588
Female Entire	14	17	1351
Female Neutered	15	22	1371
Total Male	23	49	3040
Total Female	29	39	2722
Total Entire	32	56	3803
Total Neutered	20	32	1959
Total	52	88	5762

Table 6. Details of the gender and neutering status of the hypothyroid, euthyroid and hospital groups of dogs.

The age distribution for the hypothyroid, euthyroid and hospital groups is displayed in Figure 19. The hypothyroid and euthyroid dogs were significantly ($p < 0.001$) older than the hospital group but were not significantly different to each other. However, as can be seen from Figure 19, there were a large number of young animals referred to the hospital for investigation of juvenile diseases. The exclusion of this biologically distinct group of dogs less than two years of age removed the statistically significant effect.

To identify the cause of the skewed age distribution data in the hospital group, the number of dogs less than two years of age was compared to the corresponding number above this age in each discipline. These data are displayed in Figure 20. There were highly significant ($p<0.001$) differences in the number of juvenile dogs referred to various disciplines. Orthopaedics had a significantly increased, whereas oncology, soft tissue surgery, endocrinology, and neurology, had a significantly decreased number of animals referred who were less than two years of age.

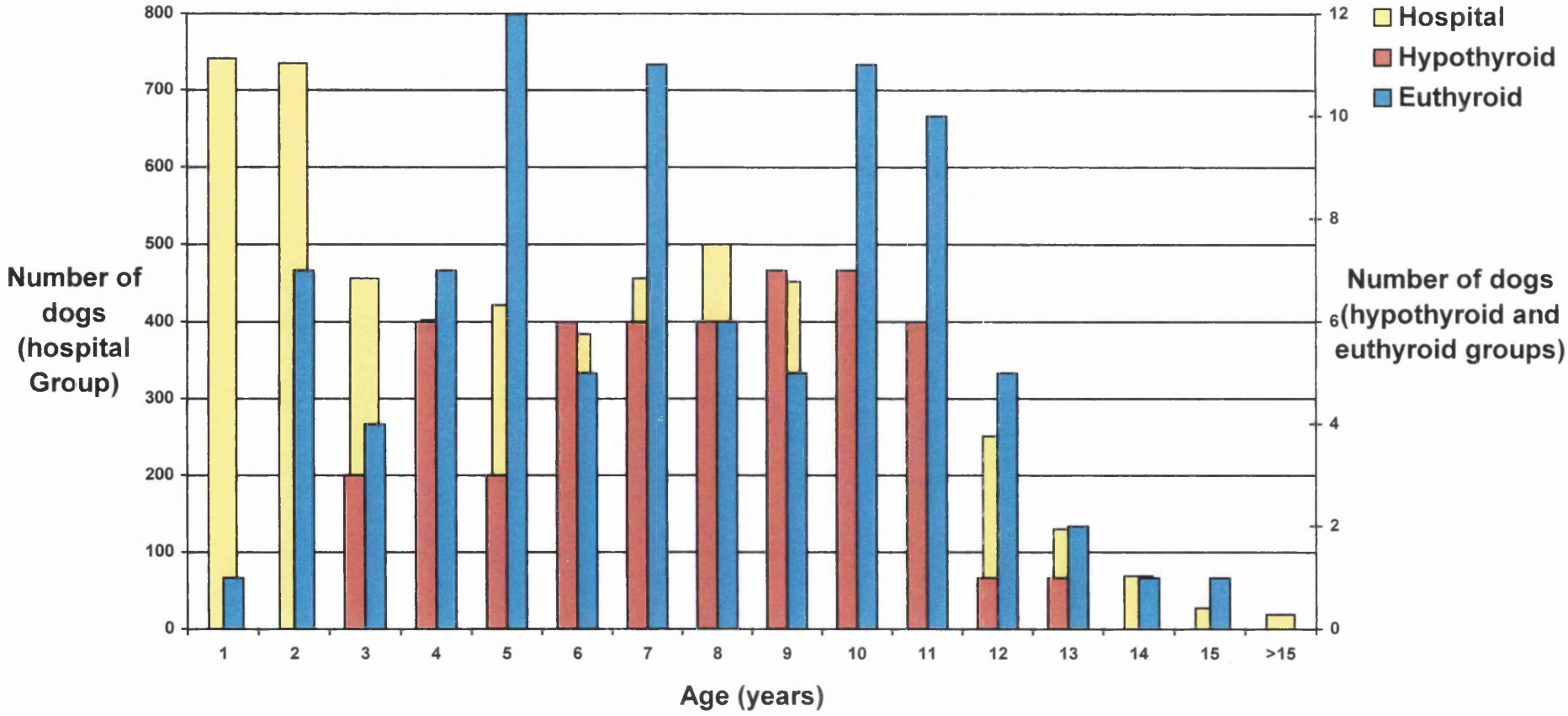


Figure 19. Age distribution of hypothyroid, euthyroid and hospital groups of dogs.

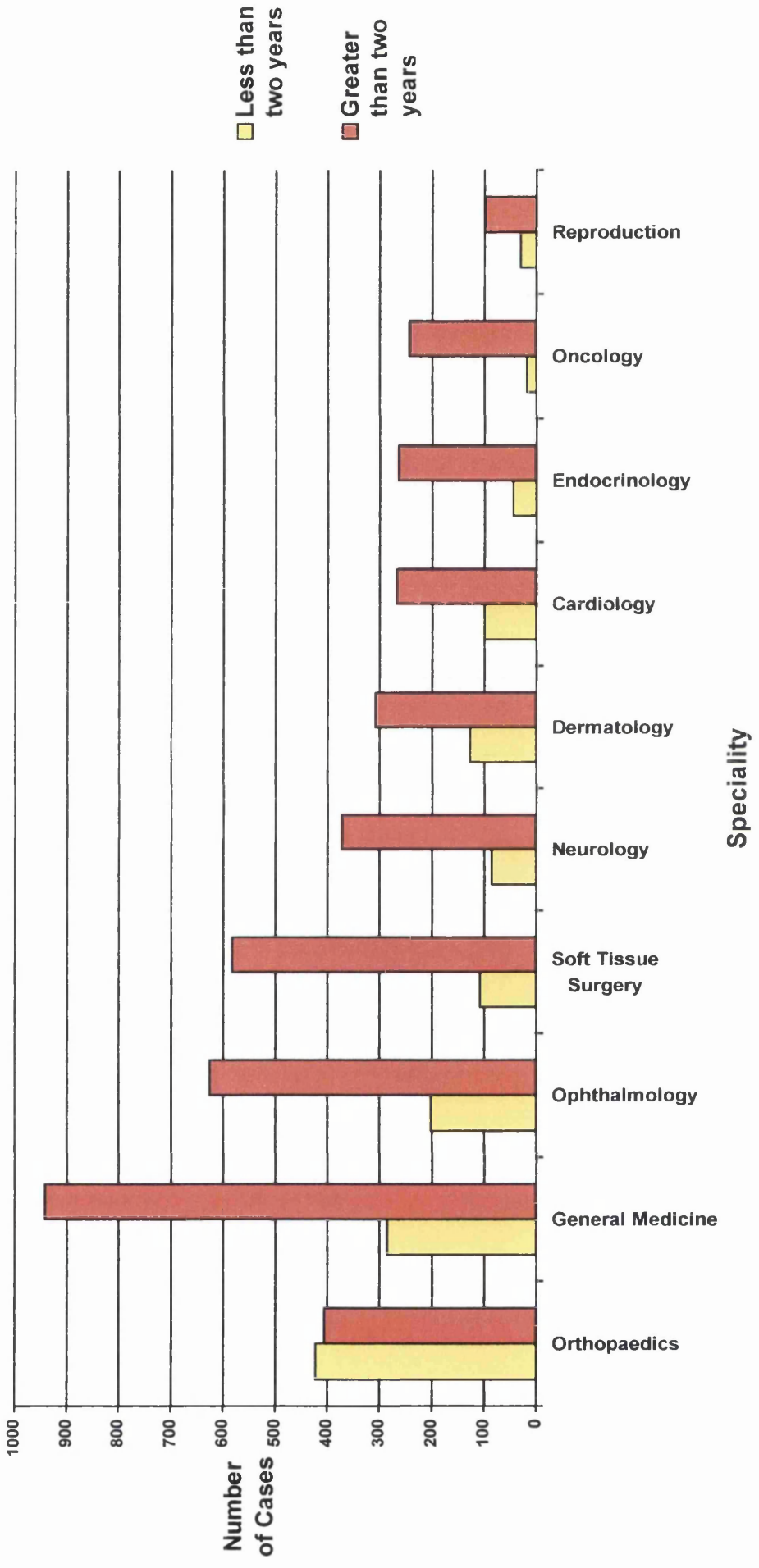


Figure 20. Relative distribution of juvenile (two years or less) dogs compared to adult dogs categorised by the speciality to which they were referred.

Clinical Findings

The most commonly reported clinical abnormalities in the hypothyroid dogs are displayed in Figure 21. The most common group of clinical features were metabolic signs, at least one of which was recorded in 44 (85 %) cases. These included lethargy (n=40, 77 %), obesity or weight gain (n=22, 42 %), exercise intolerance (n=14, 27 %), cold intolerance (n=5, 10 %), generalised weakness (n=6, 11 %) and shivering (n=3, 6 %). Dermatological abnormalities were recorded in 41 (79 %) cases and included hair thinning (n=29, 56 %) particularly affecting the flanks, tail and thighs, dry or poor quality coat (n=15, 29 %), skin hyperpigmentation (n=10, 19 %) and superficial pyoderma (n=8, 15 %). A typical example is illustrated in Figure 22. Otitis, seborrhoea, skin thickening, skin odour, nasal hypopigmentation and comedones were each recorded in fewer than 10 % of cases. Metabolic and dermatological signs were observed concurrently in 35 (67 %) dogs. Less common clinical signs included diarrhoea (n=2), neurological signs (n=3), and irregular interoestrus intervals (n=1). The neurological signs included facial nerve paralysis in two dogs and ataxia, vertical nystagmus and proprioceptive deficits in one further dog presumably related to a concurrent encephalitis of unknown aetiology confirmed at post mortem examination. Histological examination of the thyroid gland in the latter case was confirmatory of idiopathic atrophy.

Other concurrent diseases were present in 20 of the hypothyroid dogs. These included musculoskeletal disease (n=4), consisting of one case each of osteoarthritis, unilateral cranial cruciate rupture, cervical spondylopathy, patellar luxation with tarsal ligament collapse; dilated cardiomyopathy (n=3), adult onset demodecosis (n=2), ocular abnormalities (n=2), mammary neoplasia (n=2), eosinophilic bronchitis (n=1), hyperadrenocorticism (n=1) and interdigital cysts (n=1). Five (10 %) hypothyroid dogs had concurrent diabetes mellitus including one of the dogs with mammary neoplasia.

Further details of the case with concurrent hyperadrenocorticism are provided in Appendix 32.

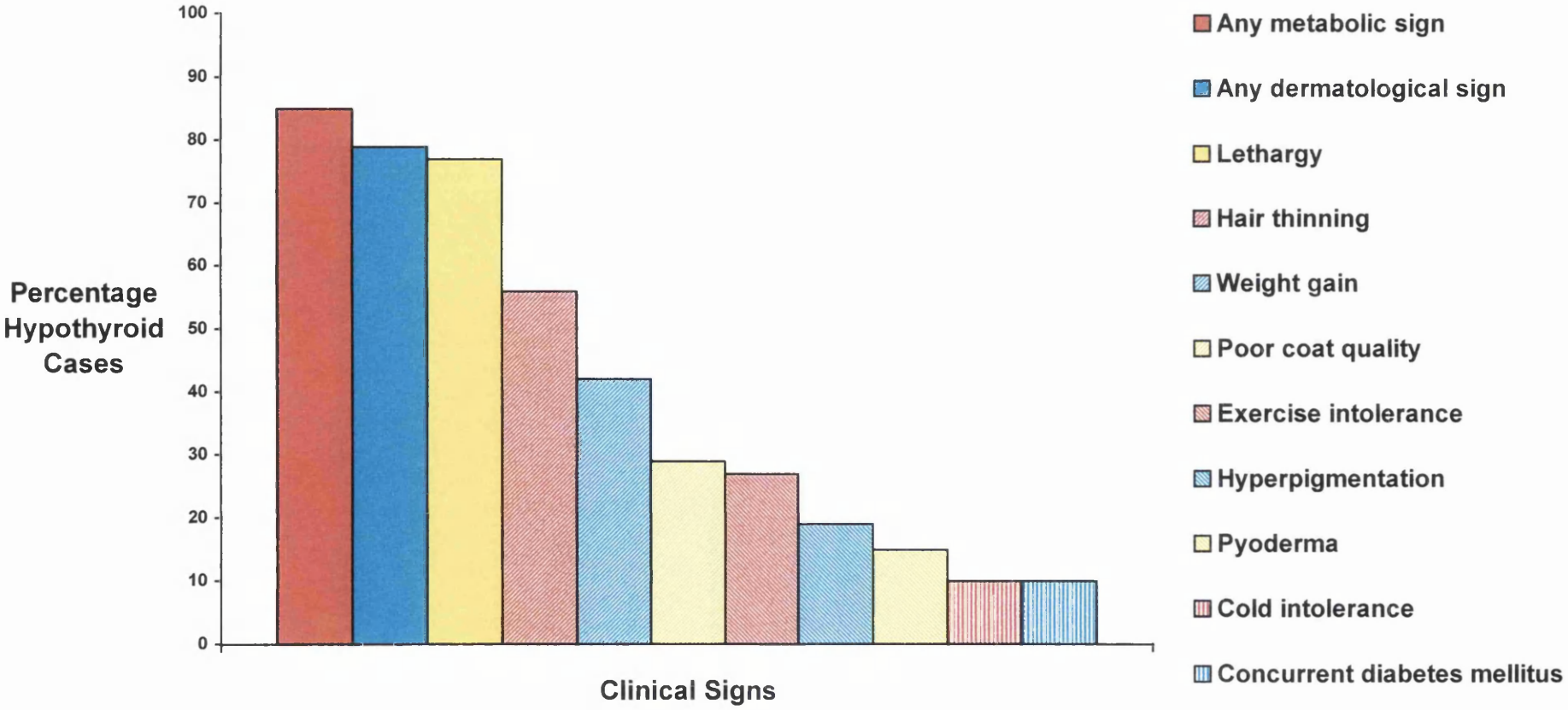


Figure 21. Distribution of the clinical abnormalities recorded in 52 dogs with confirmed hypothyroidism

Figure 22. Example of dermatological abnormalities in a dog with confirmed spontaneous hypothyroidism.

Clinicopathological Findings

The reference limits obtained by analysis of the clinicopathological data from the euthyroid group are detailed alongside the corresponding laboratory reference limits in Table 7.

Analyte	Observations	Reference limits generated from the euthyroid group		Laboratory reference limits	
		Lower reference limit	Upper reference limit	Lower reference limit	Upper reference limit
Urea	87	2.32	13.50	2.5	8.5
Sodium	87	135.45	151.85	136	159
Potassium	87	3.60	5.69	3.4	5.8
Chloride	87	99.15	124.70	95	115
Calcium	85	2.22	2.91	2.34	3
Magnesium	85	0.61	0.94	0.6	0.87
Phosphate	85	0.58	2.23	0.9	2.9
Glucose	72	4.33	13.72	3.3	6.5
Cholesterol	87	3.02	15.47	2	7
Creatinine	87	56.60	182.95	45	155
Bilirubin	85	0.00	4.00	0	10
ALKP	87	33.30	1646.80	0	230
AST	86	11.13	54.63	0	40
ALT	86	8.50	223.25	0	90
Total Protein	86	54.13	79.00	50	78
Albumin	86	27.13	43.88	29	36
Globulin	86	22.00	47.00	28	42
Creatine Kinase	60	61.18	452.40	0	150
Triglyceride	54	0.50	6.19	0.1	0.6
γ-glutamyl transferase	54	2.00	10.00	0	20
Fructosamine	82	168.25	427.70	162	310
RBC	57	5.18	7.77	5.5	8.5
Hb	57	11.94	17.50	12	18
MCV	57	61.00	73.60	60	77
MCH	57	20.80	25.96	19.5	24.5
MCHC	57	32.84	37.74	32	36
PLT	56	166.50	550.38	200	500
MPV	56	6.60	10.53	none	none
WBC	57	5.48	28.26	6	12
Neutrophil	57	3.78	22.63	3	11.8
Lymphocyte	57	0.53	3.32	1	4.8
Monocyte	57	0.08	1.69	0.15	1.35
Eosinophil	57	0.00	1.07	0.1	1.25

Table 7. Biochemical and haematological reference limits generated from the euthyroid group of dogs. Laboratory reference limits are also shown for comparison. See Appendices 2 and 4 for key and units.

The most common clinicopathological abnormalities in the hypothyroid and euthyroid groups when compared with the laboratory reference limits are summarised in Table 8. Examination of these results reveals a similar pattern of clinicopathological changes in both these groups. Elevated concentrations of triglycerides and cholesterol were the most diagnostically sensitive routine tests occurring in 30 of 34 (88 %) and 40 of 51 (78 %) hypothyroid and 45 of 54 (83 %) and 40 of 87 (46 %) euthyroid dogs, respectively. However, these parameters were associated with poor diagnostic specificity (0.17 and 0.54, respectively). The most diagnostically efficient abnormalities were reduced RBC count (0.69) and elevated fructosamine concentration (0.68) which occurred in 19 of 47 (40%) and 19 of 44 (43 %) hypothyroid, and 13 of 84 (15 %) and 15 of 82 (18 %) euthyroid dogs, respectively.

Analyte	*Hypothyroid dogs*			*Euthyroid dogs*						
	Number tested	**Median (SIR)**	**No. (%) outwith reference limits**	**Number tested**	**Median (SIR)**	**No. (%) outwith reference limits**	**Reference range**	**Diagnostic sensitivity**	**Diagnostic specificity**	**Diagnostic efficiency**
Triglyceride	34	1.5 (2.6)	30 (88)	54	1.2 (1.7)	45 (83)	0.1 - 0.6	0.88	0.17	0.44
Cholesterol	51	10.3 (7.0)	40 (78)	87	6.9 (4.0)	40 (46)	2.0 - 7.0	0.78	0.54	0.63
Fructosamine	44	300 (256)	19 (43)	82	249 (55)	15 (18)	162 - 310	0.43	0.82	0.68
ALKP	51	138 (297)	18 (35)	87	138 (272)	28 (32)	0 - 230	0.35	0.68	0.56
RBC	47	5.6 (1.2)	19 (40)	84	6.5 (1.2)	13 (15)	5.5 - 8.5	0.40	0.85	0.69
CK	34	100 (96)	12 (35)	60	113 (101)	21 (35)	0 - 150	0.35	0.65	0.54

SIR semi-interquartile range

Table 8. Summary of the abnormal biochemical and haematological results obtained from hypothyroid and euthyroid dogs compared with the laboratory reference range. See Appendices 2 and 4 for key and units.

Results of comparison of the routine hypothyroid clinicopathological results with the reference limits generated from the euthyroid group are displayed in Table 9. Using this approach, the most frequent abnormalities in the hypothyroid group were elevated serum cholesterol in 10 (20 %), and γ-GT concentration in six (18 %), and decreased RBC in 14 (30 %), haemoglobin in 13 (30 %) and neutrophil count in eight (17 %) of the cases tested.

Statistically significant differences in sodium, chloride, calcium, cholesterol, creatinine, AST, globulin, fructosamine, RBC count, Hb concentration, MCH, MCHC and eosinophils were found in the hypothyroid dogs compared with the euthyroid group (Table 10).

Analyte	Number tested	Median (SIR)	No. (%) outwith reference limits		Reference limits
			Above	Below	
Cholesterol	51	10.3 (7.0)	10 (20 %)	1 (2 %)	3.0 - 15.5
γ-GT	34	5.0 (3.0)	6 (18 %)	2 (6 %)	2.0 - 10.0
RBC	47	5.6 (1.2)	1 (2 %)	14 (30%)	5.2 - 7.8
Haemoglobin	43	12.9 (2.3)	0	13 (30%)	11.9 - 17.5
Neutrophils	47	7.4 (5.4)	2 (4 %)	8 (17 %)	3.8 - 22.6

SIR semi-interquartile range

Table 9. Abnormal biochemical and haematological results in hypothyroid dogs compared to the reference limits derived from the euthyroid group of dogs in which hypothyroidism had been suspected but excluded. See Appendices 2 and 4 for key and units.

Analyte	Hypothyroid (median (SIR))	Euthyroid (median (SIR))	p-value
Sodium	144 (4)	146 (4)	0.0004
Chloride	110 (7)	112 (6)	0.0038
Calcium	2.7 (0.3)	2.6 (0.3)	0.01
Cholesterol	10.3 (7.0)	6.9 (4.0)	<0.0001
Creatinine	105 (37)	90 (27)	0.0013
AST	26 (15)	23 (12)	0.0382
Globulin	33.0 (6.7)	29 (7.2)	0.0033
Fructosamine	299 (84)	249 (55)	0.0025
RBC	5.6 (1.2)	6.5 (1.2)	<0.0001
Hb	12.9 (2.3)	15.4 (2.0)	<0.0001
MCH	23.0 (1.8)	23.8 (1.9)	0.02
MCHC	34.5 (1.9)	35.3 (1.6)	0.0016
Eosinophils	0.19 (0.32)	0.33 (0.34)	0.0279

SIR semi-interquartile range

Table 10. Statistically significant differences in the routine biochemical and haematological results between hypothyroid and euthyroid groups of dogs. See Appendices 2 and 4 for key and units.

5.5 DISCUSSION

A fundamental objective in the epidemiological study of any disease is the identification of biological and environmental associations with the disease. In some cases a solitary "cause" of the disease may be identified. Alternatively, a variety of factors, all of which increase the likelihood of the occurrence of the disease may be identified. The identification of a biological characteristic or trait associated with a particular condition requires demonstration that the trait is significantly over or under-represented in the diseased compared to the non-diseased "control" population. Clearly, with the exception of the disease being studied, the control group should be similar in all other respects to the diseased group. Given an adequate quantity of data, this "matching" of groups is achievable for certain biological characteristics such as age, breed and sex. However, in practice matching certain other variables such as nutrition, local environmental conditions, previous diseases or therapies is unrealistic due to inadequate data. It should be clear that by matching diseased and control groups for a particular variable, the association of that variable with the presence of disease can no longer be evaluated.

A particular problem which exists in nearly all clinical research studies, results from biases within the population which is investigated for disease, compared to the population at risk from disease. For example, it would be naive to assume that the signalment data for cases presenting to veterinary surgeons reflected the true distribution in the general canine population. Social, economic and disease factors all play a role in determining if an animal is submitted by its owner for veterinary investigation. Therefore, the frequent presentation of one particular breed of dog with any specific disease does not necessarily imply that the breed is prone to that disease in the general canine population. Such a pattern may result from disproportionate submission of that breed to veterinary surgeons for investigation. This biasing effect of dogs at risk of disease compared to dogs with the opportunity for veterinary investigation is particularly marked in the case of a referral population since only a specific subset of diseased cases are referred to a second opinion clinic. This complicating feature of epidemiological analysis is important to appreciate if the results of clinical study are not to be misinterpreted.

The use of referral data is further complicated by the influence that specialties within the referral centre exert on the make-up of the control category. Heavy biases with respect to age and breeds of dogs are particularly common within certain disciplines and may affect the eventual analysis. This will affect the apparent association of age, sex, breed etc. amongst the group of dogs under study (Graham, 1995). This feature was clearly

demonstrated in the present study, with a high percentage of referred cases being juvenile animals. The distribution of cases within a referral control population should be examined and if necessary any such biases removed during the selection of the control group. This was performed in the present study and highlighted that the statistically significant effect of age between both the hypothyroid and euthyroid groups, compared with the hospital group was simply a consequence of this type of error. However, as evidenced by the results detailed in Appendix 31, the practical difficulties associated with completely resolving this issue can become truly prohibitive. A comprehensive study of this aspect of referral epidemiology alone would be worthy of a dedicated study.

Despite these problems, in clinical research a pragmatic approach to the selection of control groups is usually necessary. In practice, most studies use those cases which were confirmed with diseases other than the one of interest during the equivalent time period as the control group (Milne & Hayes, 1981; Panciera, 1994). To an extent this allows comparison of "like with like" and assists in identifying biological associations with disease. This approach was adopted in the present study and was found to be practical and achievable. The results are therefore directly comparable with other equivalent studies. However, without a knowledge of the biological phenotype of the general canine population in comparison to the referral population, extrapolation of the results must be performed cautiously. The study by Nesbit *et al.*, (1980) is an example of these problems as they relate to canine hypothyroidism in particular. In that study hypothyroidism was reported to be most common in doberman pinschers, great Danes, poodles, and schnauzers. However, no information was provided regarding the distribution of these and other breeds within the non-hypothyroid population examined. Consequently the conclusions on breed predisposition to hypothyroidism are questionable since the apparently high prevalence of these breeds may simply have been the result of a high prevalence within the referral population itself. There is a surprising absence of even basic demographic data on the number and signalment of dogs within the UK and this further hinders the epidemiological analysis of diseased populations.

The external group was selected and included in the present study for two reasons. Firstly it was used for directly comparing to the breed data obtained from the hypothyroid dogs. Secondly, comparison of the hospital group with the external group was desirable to provide some indication of the "trueness" of the distribution of referred dogs in relation to the relative number of those breeds in the general population. No information of the relative numbers of males and females was available from the KC. It was accepted that the

external control group data were subject to potential error from a variety of causes. These included

- changing trends in breed popularity influencing the number of new KC registrations relative to the total number of that breed in the general population
- any demographic variation specific to central and western Scotland from where the majority of the hospital group was referred
- the inability to assess any effect of crossbreeds as a specific "breed type"

It was considered that in reality the first two criteria were unlikely to have an undue effect on the results. These assumptions were supported by the "first twenty tables" published by the KC. These list the 20 most commonly registered breeds each year, and during the period of study showed very little annual variation suggesting relatively stable breed popularities. Also, the fairly similar breed rankings of the external and hospital groups of dogs, suggests that the local geographical breed demographic composition is similar to that of the UK as a whole. The third criteria was recognised as a genuine limitation but accurately addressing it was considered to be outwith the scope of this study. Nevertheless some estimation of this factor was desirable to allow evaluation of any influence that pedigree versus non-pedigree status may have on the development of hypothyroidism. The most recent data published by the Pet Food Manufacturers' Association (PFMA) (PFMA Profile, 1998), estimated that approximately 60 % of dogs within the UK are pedigree whilst the remainder are crossbreeds. Consequently this estimate was used as a basis from which the distribution of pedigree and non-pedigree dogs within the hypothyroid and control groups could be evaluated.

The data initially obtained from the KC was grouped according to the breeds used in the KC classification scheme. However, in practice, when first referred to the DVCS many of the hypothyroid or various control dogs were only broadly classified with regard to their breed. For example a referred German spitz may have only been classified as "German spitz" with no reference to, in this case, either Klein or Mittel. Consequently the breed classification of the hypothyroid and control animals was not consistent with that of the KC for all types. Where appropriate, the KC data were combined to generate groupings which corresponded to that of the other groups of dogs in the study. For example all spaniels, whether cavalier, cocker or springer varieties, were combined and considered as a single breed type for statistical analysis. This grouping risked masking predispositions

present within one variety of a particular breed type which may not be present in the others. However, the grouping was considered necessary to allow any valid comparisons with the other dogs in the study. In addition, the grouping of breeds into "types" allowed the broad genetic makeup of groups to be maintained, whilst increasing the size of the groups therefore making them more suitable for useful statistical analysis. This was particularly relevant in the hypothyroid group where the number of animals of each breed type was relatively small. Whilst not a perfect solution, this approach was considered a practical and reasonable one.

A second problem associated with the quality of data collection in all cases referred to the DVCS resulted from poor recording of cases as pedigree or non-pedigree types. In some cases, dogs were categorised as being non-pedigree but of predominantly one type for example "rottweiler-cross". However, for many cases, only the predominant breed type would be recorded potentially misclassifying a substantial number of non-pedigree dogs as pedigree. This was recognised as a genuine limitation of the present study when evaluating the effect of pedigree on the development of hypothyroidism, since accurate classification of pedigree status could not always be relied upon. The approach adopted for the purposes of this study, was to classify all those cases recorded as crossbreeds as "non-pedigree" and in the remaining animals, in which a single breed was recorded, as "pedigree". This approach immediately introduces a degree of error into subsequent statistical analyses and artificially increased the apparent referral of pedigree animals. However, the advantage of this approach was twofold. Firstly it increased the numbers of cases within each breed or breed-type category as discussed above, making the data more amenable to statistical analysis. Secondly, the use of categories in which a single breed was identified as the predominant, if not the sole breed, maintained the broad genetic makeup of the categories as pedigree-type or non-pedigree. Given the unavoidable limitations inherent within the hospital data recording system, as with breed type classification it was felt that the advantages associated with this solution outweighed the disadvantages.

In the present study there was no apparent effect of either sex or neutering status on the likelihood of being hypothyroid. This is similar to the results of Peterson *et al.*, (1997) but is in contrast to the findings of Milne & Hayes, (1981); and Panciera, (1994). The loose diagnostic criteria employed by Milne & Hayes, (1981) for the confirmation of hypothyroidism casts doubt on their conclusions. However, like the present study, Panciera, (1994) also used TSH response testing to confirm hypothyroidism and the reasons for the discrepancy with that study is unclear. This variation may reflect a

difference in the populations under study although both Panciera, (1994) and Peterson *et al.,* (1997) investigated dogs in the United States of America (USA) with differing results. Irrespective of any influence that gender and neutering status may have on the development of hypothyroidism, it is clear that in the dog at least, both males and females whether sexually entire or neutered are frequently affected by hypothyroidism.

The results of this study confirmed that hypothyroid dogs are significantly older than the general hospital referral population. However, a more detailed analysis of the distribution of dog ages within the referral population revealed a strongly bimodal pattern with a large number of young cases. This peak of juvenile referrals is not surprising since many cases are referred for investigation or treatment of congenital or juvenile developmental conditions. This was confirmed by demonstrating a statistically significant variation in the age distribution of dogs referred to the varying disciplines and is consistent with the nature of many of the diseases seen within each of these groups. This problem highlights one potential bias introduced by use of a referral population as a control group. However, as already discussed, it is rare for published studies to take these effects into account (Milne & Hayes, 1981; Kaelin *et al.,* 1986; Panciera, 1994). It is clear from the results of the present study that if not identified and eliminated, this type of influence may artificially create or inadvertently mask genuine biological phenomena.

The exclusion of this biologically distinct group of juvenile individuals from the referral controls confirmed that there was in fact no difference in age between hypothyroid dogs and those referred for other conditions. Similarly there was no difference in the age distribution of the hypothyroid and euthyroid dogs. Based on these results the age of a dog is therefore not particularly useful in the investigation of hypothyroidism. However, the results do indicate that juvenile animals (less than two years of age) are rarely affected, and the diagnosis is equally likely any time between approximately four and 11 years of age. Since no breeds or breed types were identified as being predisposed to hypothyroidism, stratification of either high-risk or low-risk breeds to compare the relative risk in varying age groups was not considered appropriate. Comparison of this feature with the report by Milne & Hayes, (1981) was therefore not possible.

The most commonly affected breed types in the present study were retrievers, spaniels, terriers, crossbreeds and dobermans. This is broadly similar to the most recent reports of the breed distribution of hypothyroid dogs, most of whom confirmed hypothyroidism using reliable criteria (Panciera, 1994; Feldman & Nelson, 1996; Peterson *et al.,* 1997). The results vary from those reported in earlier studies most of which used

more dubious criteria for case classification (Nesbitt *et al.,* 1980; Milne & Hayes, 1981). However, there was no significant difference between the breed distribution of the hypothyroid cases with either the euthyroid or hospital control groups. The apparent over-representation of breed types such as retrievers and spaniels may therefore simply reflect their popularity within the referral population.

There was a marked bias in the distribution of certain breeds in the referred population compared to their number in the external group, with collies, terriers, rottweilers and doberman pinschers being significantly over-represented in the hospital group. Again this is partly due to the nature of the referral population since a number of disciplines include diseases with marked apparent breed-predispositions which bias the control data. This further exemplifies the potential difficulties associated with using a referral population as a control group.

Whilst a true estimation of the relative distribution of pedigree and non-pedigree dogs within the general population is difficult to establish with any degree of accuracy, it certainly seems unlikely to be reflected by the individuals in this study. The number of pedigree dogs referred approached 90 % of all cases seen and this was responsible for the difficulties in further statistical analysis. However, even the PFMA estimated prevalence of 60 % pedigree dogs in the general canine population may well be higher than the true figure. The results of this study suggest that pedigree dogs are genuinely more frequently referred than are non-pedigree dogs. However, the present study has certain fundamental limitations discussed above, and no well controlled studies have previously accurately investigated this subject. Nevertheless the present study has identified this as an area worthy of additional investigation.

There was no significant difference in the pedigree status of dogs in the hypothyroid, euthyroid and referral groups. However, the relatively large number of pedigree compared to crossbreed dogs in the control groups makes analysis of this variable prone to statistical interference. In addition due to the variability in the quality of data recording regarding the true pedigree status of the dogs in this study, the definition of pedigree used for this study is fairly loose as discussed previously. Consequently, these dogs may in fact be more accurately considered as "purebred-type" animals. The consequence of both the classification protocol used, and the marked asymmetry in numbers of "pedigree" and "non-pedigree" dogs ultimately made the evaluation of this factor on the development of hypothyroidism difficult to assess. The absence of any apparent breed predisposition may suggest that there is no strong pedigree effect on the

development of the disease but further studies are required. The subject of pedigree status on development of hypothyroidism is discussed in Chapter 7.

Metabolic signs were the most common clinical signs observed being reported in 85 % of cases. These included lethargy, obesity or weight gain, and exercise intolerance. These figures are similar to those reported by Kaelin *et al.,* (1986) but suggest that metabolic abnormalities are in fact more prevalent than reported in most previous studies (Nesbitt *et al.,* 1980; Panciera, 1994; Feldman & Nelson, 1996). Whilst the differences may reflect the varying populations studied, other explanations include the severity of disease which may influence the signs observed (Feldman & Nelson, 1996).

Dermatological abnormalities were also particularly common occurring in 79 % of cases. The most common of these were alopecia, poor coat quality, hyperpigmentation, and superficial pyoderma. These are similar to the findings of previous studies (Nesbitt *et al.,* 1980; Kaelin *et al.,* 1986; Panciera, 1994). Of particular interest amongst the hypothyroid group was the high percentage with both metabolic and dermatological abnormalities (67 %). This combination therefore proved to be a useful marker for the presence of the disease and based on these results their combined presence should certainly prompt further investigation of hypothyroidism.

Other clinical signs were considerably less common than either metabolic or dermatological abnormalities. Neurological signs occurred in three dogs but in two of them the relationship of these signs to hypothyroidism was made more difficult to interpret by concurrent disease. In the third case, with facial nerve paralysis, a good clinical response occurred following appropriate thyroid hormone replacement therapy. A number of hypothyroid dogs had concurrent diseases which were not considered likely to be related to the thyroid disease. Musculoskeletal disease and neoplasia occasionally occurred but were considered coincidental.

The role of hypothyroidism in the three cases with dilated cardiomyopathy is interesting even if not entirely clear. From the study reported by Calvert *et al.,* (1998) it seems clear that hypothyroid dogs are not more likely to develop dilated cardiomyopathy than euthyroid dogs. However, finding this abnormality in three of 52 affected cases was higher than expected. One explanation may be that the study of Calvert *et al.,* (1998) evaluated only doberman pinschers, a breed known to be predisposed to cardiomyopathy. This population bias will potentially decrease any effect of thyroid disease as a causative factor. Additionally, the three affected dogs in the present study consisted of a Labrador

retriever, English springer spaniel and a Hungarian viszla and studies with a larger number of various breeds of dog would undoubtedly be of considerable value.

The presence of concurrent diabetes mellitus in 10 % of hypothyroid cases was of particular interest and suggestive of autoimmune polyglandular disease in these individuals (Graham, 1995; Feldman & Nelson, 1996; Dixon, 1998). The investigation of these cases in more detail is outlined in Chapter 7.

Much of the discussion relating to the selection of appropriate cases to use as control animals in the investigation of the epidemiological features of disease, applies equally to the study of clinicopathological abnormalities. Traditionally the routine biochemical or haematological evaluation of diseased animals has assisted with the confirmation or exclusion of disease by comparison of the observed values with the reference distribution. This is the distribution of reference values obtained from a range of putatively "healthy" individuals, the reference sample group, who are considered to be representative of the entire reference population. However, the use of clinicopathological tests in this way is complicated for a number of reasons.

"Normal" biological variation exists within the state of "normality" and this should be considered during selection of a reference sample group. Factors such as the breed, age, sex, reproductive state, dietary variation, state of nutrition, housing, season etc. may all potentially influence certain test results and should theoretically be taken into account. However, the practicalities of accounting for all potentially influential variables can rapidly become prohibitive, with the financial and labour requirements often being the most constraining factors. Consequently as with clinical control groupings, a pragmatic approach to the selection of the reference sample group is generally employed. In fact, the reality is that many reference sample groups are composed of small numbers of animals, frequently of a single breed housed in similar environmental conditions. Consequently the range of samples used for calculation of the reference limits is frequently very limited. An obvious example of this is the widespread use of beagles in veterinary research which are often the source of "normal" blood samples.

A second and more fundamental problem exists in relation to the use of conventional clinicopathological control groups. It is traditional to compare observed values with the reference limits derived from a supposedly normal reference sample group. Therefore by definition the identification of an "abnormal" value only in fact serves to distinguish diseased animals from healthy animals. However, the purpose of clinicopathological analysis is generally to support or refute a clinical suspicion or diagnosis

and it is certainly rare that samples are analysed to differentiate "health" from "disease" per-se. This misuse of reference limits is either widely ignored or misunderstood. When trying to differentiate between particular diseases as is most often the case in practice, the most appropriate reference sample group is in fact those cases in which a particular disease is suspected but proven not to be present. These animals represent the cases which in practice must be segregated from patients with the disease in question.

Most practical problems arise when examining analytes which can be affected by multiple types of disease or pathological processes. For example increased circulating cholesterol concentrations are associated with a variety of disease states including diabetes mellitus, hypothyroidism, hyperadrenocorticism, hepatic disease, and hypoalbuminaemia. The specificity of hypercholesterolaemia for any one of these diseases, some of which are clinically very similar, is correspondingly poor.

When compared with the laboratory reference ranges, the abnormalities obtained from the hypothyroid dogs gave results broadly in keeping with previous studies. Cholesterol was elevated in 78 % of the hypothyroid dogs tested which is similar to the studies by Kaelin *et al.,* (1986); Peterson & Ferguson, (1989); and Panciera, (1994). Circulating fructosamine concentration was increased in 43 % of the hypothyroid group which is a previously unreported finding in dogs. This abnormality is recognised in human hypothyroid patients whilst hyperthyroid humans typically have subnormal fructosamine concentrations (Ford, Lim & Crooke, 1987; Waterson & Mills, 1988; Sako, Umeda, Hashimoto, Haji & Nawata, 1989). The abnormalities in both situations are attributed to a change in rate of protein turnover rather than any effect on glycaemic control itself. The diagnostic specificity of fructosamine for hypothyroidism (0.82), following the exclusion of dogs with known diabetes mellitus, was surpassed only by the RBC count (0.85). Based on the results of this study, and given the relative ease with which diabetes mellitus can usually be excluded, the addition of fructosamine estimation as a screening test when investigating suspect hypothyroidism is likely to be of at least as much value as the other routine biochemical and haematological analytes currently available. Creatine kinase was increased in 35 % of affected dogs which is slightly more than 10 % reported by Jaggy *et al.,* (1994) and 18 % reported by Panciera, (1994). However, in the present study, the same percentage of euthyroid dogs had increased CK concentrations and the analyte was therefore of no predictive value. Forty percent of the hypothyroid dogs were anaemic which is similar to previous reports (Kaelin *et al.,* 1986; Panciera, 1994; Feldman & Nelson, 1996). Although the RBC count was also depressed in 15 % of the euthyroid

group, this analyte followed closely by fructosamine and cholesterol was overall the most efficient routine parameter at separating hypothyroidism from clinically similar non-thyroidal illness.

It is clear from the results presented that a substantial number of euthyroid dogs with various non-thyroidall illnesses had routine biochemical and haematological parameters well outwith typical laboratory reference limits. This included many of the analytes traditionally associated with abnormal values in hypothyroidism including cholesterol, CK, and RBC count. The effect of this was that the specificity of some of these measurements for predicting the presence of hypothyroidism was particularly poor. The reference distribution generated from the euthyroid group results was correspondingly wide. Comparison of the hypothyroid results with the reference limits derived from these data served to clearly illustrate the very poor specificity of most routine parameters for hypothyroidism. This feature of the present study emphasises the errors that are widely ignored in the comparison of results with so-called "normal" populations in which the control samples are collected from healthy patients. It is clear from the present study that such comparisons do not produce a true reflection of the predictive value of these parameters for hypothyroidism and the same is likely to be true for most diseases.

The use of the euthyroid group reference limits, allowed the identification of routine parameters which were significantly different between those dogs with hypothyroidism, and those cases with clinically similar illnesses. Although the results presented confirm a large number of variables are statistically different between the groups, only those analytes with values outwith the reference limits are likely to be of diagnostic use in practice. Using this novel approach, elevations in cholesterol and γ-GT, and decreases in RBC count, haemoglobin and neutrophil count were the most common abnormalities associated with hypothyroidism. Although the control data needed to generate these results is clearly demanding in terms of clinical material, accurate case classification, and time requirements including statistical analysis, their improved diagnostic specificity is highly desirable. The future use of this type of control data should be encouraged from both academic institutions and commercial diagnostic laboratories.

CHAPTER 6

TREATMENT AND THERAPEUTIC MONITORING OF HYPOTHYROIDISM

6.1 LITERATURE REVIEW

Treatment of Hypothyroidism

Indications and Contraindications

The principal indication for THRT in dogs is the treatment of spontaneous hypothyroidism. There are few indications for bilateral thyroidectomy in the dog and therefore little is known about the requirement for THRT after such a procedure. It may be that as in the cat, where bilateral thyroidectomies are frequently indicated for the treatment of hyperthyroidism, accessory thyroid tissue may be activated, thereby obviating the need for THRT (Swalec & Birchard, 1990). However, cTSH may have growth stimulatory properties on thyroid tumours and therefore could play a role in subsequent recurrence (Verschueren, Rutteman, Vos, van Dijk & de Bruin, 1992). Therefore, THRT has been recommended following surgical removal of thyroid carcinomas in dogs, even when unilateral, on the basis that such therapy inhibits production of cTSH. THRT has also been recommended following transphenoidal hypophysectomy, a technique investigated for the treatment of pituitary dependent hyperadrenocorticism in dogs (Meij, Mol, van den Ingh, Bevers, Hazewinkel & Rijnberk, 1997).

The role of THRT in NTI has been studied in dogs as well as humans. Brent & Hershman, (1986) evaluated the effect of intravenous T_4 replacement therapy on mortality in 11 of 23 hospitalised human patients with severe NTI and subnormal circulating total T_4 concentration. Prognosis was not altered by therapy, and mortality was similar in both the treated (73 %) and untreated (75 %) patients. DAlecy, (1997) evaluated the effect of intensive THRT immediately following controlled experimental cardiac arrest in dogs. A neural protective effect was demonstrated in those dogs receiving the THRT compared to control cases. The author concluded that thyroid hormone-mediated improvement in systemic oxygen consumption and tissue delivery may have contributed to the protective effect. However, whilst intensive THRT regimes such as these have been shown to be beneficial in experimental situations, there remains little evidence to support the use of THRT to "treat" the subnormal thyroid hormones encountered in the more typical NTI encountered in veterinary practice (Panciera, 1997a). The reduced peripheral conversion of T_4 to T_3, and the reduced secretion of TSH documented in NTI likely serve to reduce

metabolic demand, particularly catabolic activity. Over-riding this protective mechanism is probably unwise.

Physiological Considerations

A review of normal thyroid gland and thyroid hormone physiology and metabolism is provided in Chapter 4 and those comments remain relevant to a discussion on therapy for hypothyroidism.

Ideally, the goal of treatment in the hypothyroid patient is to mimic the secretory pattern of the healthy thyroid gland thereby restoring the patient to a state of physiological normality. However, the only practical method for implementing chronic THRT in dogs is by oral administration of thyroid hormone preparations. Such "bolus" type dosing is thus a relatively crude alternative when compared with the normal pulsatile secretion of the thyroid hormones (Kemppainen & Sartin, 1984). Therefore, achieving a state of physiological normality is an impractical objective. A more reasonable goal is the elimination of routine clinicopathological and clinical abnormalities associated with the disease, thereby restoring the animal back to "clinical" health.

As discussed in Chapter 4, thyroid hormones circulate largely bound to plasma proteins. However it is the free hormone which is the metabolically active fraction available for entry into cells. The protein bound moiety serves to distribute the hormone throughout the circulation and being in dynamic equilibrium with the free moiety, acts as a reservoir of hormone. This applies in both healthy dogs and those receiving THRT, and provides a buffering mechanism which protects against rapid increases or decreases in free thyroid hormone concentrations. Certain organs, especially the liver and the kidneys can also store and rapidly exchange large quantities of thyroid hormones with the circulation. This provides additional buffering capacity (Ferguson, 1986). These normal physiological mechanisms fortuitously assist in delivering a relatively even supply of free thyroid hormones and therefore help reduce the "peak and trough" characteristics of THRT.

As previously discussed, circulating free T_4 constitutes approximately 0.1 % of the total T_4 concentration in dogs, whereas approximately 1 % of total T_3 circulates unbound. Free T_3 therefore has an increased volume of distribution compared to T_4 which is more tightly contained within the circulation. This promotes a faster exit of T_3 from the vascular compartment into the peripheral tissues and as a consequence T_3 is metabolised more rapidly. The half life of T_3 in dogs (five to six hours) is correspondingly shorter than T_4 (10

to 16 hours). The biological activity of T_3 is considerably greater on a per-molar basis than that of T_4. Whilst this may imply that therapy for hypothyroidism would be more efficiently achieved by the administration of T_3, the shorter half life requires that this form of therapy be administered more frequently. In addition, the potential for thyrotoxicosis due to overdosage is greater with T_3-containing products (Panciera, 1997a). Also, most circulating T_3 in dogs normally originates from peripheral deiodination of T_4. This step allows maintenance of both normal total and free T_4 and T_3 concentrations in the healthy individual assuming an adequate supply of T_4. However, administration of exogenous T_3 circumvents this enzymatic step which usually functions normally in hypothyroid dogs. The use of T_4-containing preparations is therefore preferable to those containing only T_3 when instituting THRT (Refsal & Nachreiner, 1997b; Panciera, 1990b).

The modes of action of the thyroid hormones has been reviewed in Chapter 4. Broadly speaking, the metabolic activity of free T_3 and free T_4 results from combination of hormone with cell membrane and nuclear receptors. The molecular effect usually results from gene transformation or protein synthesis and therefore the overt clinical responses to thyroid hormone supplementation are diverse, including tissue growth, maturation and repair. Consequently, there is a lag phase between thyroid hormone induced molecular changes which effectively occur instantaneously, and the occurrence of clinical changes which may take place over weeks or months.

Determinants of Clinical Efficacy of Therapy for Hypothyroidism

The ultimate determinants of the efficacy of THRT depend upon drug administration and absorption characteristics, and peripheral and cellular hormone metabolism. It is estimated that the total daily secretion of T_4 in the dog is approximately 0.0194 nmol/kg (Ferguson, 1986). However when Hulter, Gustafson, Bonner, Toto & Mackie, (1984) administered this dose of hormone intravenously or subcutaneously to thyroparathyroidectomised dogs, the circulating hormone concentrations remained severely depressed. In fact it was not until doses four times greater were administered that normal blood concentrations were achieved. No definitive explanation for this finding was provided by the authors but possible causes include an effect of hypothyroidism or hypoparathyroidism on serum binding capacity for thyroid hormones, local hormone degradation at the site of administration or erroneous estimation of the true thyroid hormone production rate in healthy dogs. Irrespective of the mechanism, if normalisation of circulating thyroid hormone concentrations is a requirement when treating canine hypothyroidism, greater

doses of hormone supplement are required than suggested by the daily production rates in healthy dogs.

An additional variable to be considered after oral administration of hormone, is the effect of gastrointestinal absorption. In humans, approximately 50-80 % of T_4 and nearly 100 % of T_3 is absorbed following oral administration. However, variation occurs as a result of variable intraluminal contents such as plasma proteins, intestinal bacterial load and other dietary components. These may bind free hormones and therefore inhibit their absorption. In the dog, it is estimated that only between 10 % and 50 % of orally administered T_4 is absorbed from the intestinal tract therefore increasing the required therapeutic dose when given by this route (Ferguson, 1986). Further increases are likely to be required in dogs with concurrent small intestinal malabsorption diseases (Panciera, 1997c).

As previously discussed, peripheral conversion of T_4 to T_3 is responsible for production of most of the circulating T_3 in dogs. This process is equally important in hypothyroid dogs being supplemented with exogenous T_4. In humans, the conversion process is impaired in most chronic disease states, including starvation, diabetes, hepatic and renal diseases. A number of dogs in which inhibition of peripheral deiodination of T_4 to T_3 is suspected have been reported. However, although rarely recognised in humans (Kleinhaus, Faber, Kahana, Schneer & Scheinfeld, 1988), this is yet to be documented as a spontaneous abnormality in dogs, and is usually due to the presence of concurrent illness or drug therapy (Ferguson, 1986; Panciera, 1990b). It has been suggested that hypothyroid dogs with concurrent illnesses or those receiving certain medications may therefore require greater doses of T_4 supplementation to achieve an optimal clinical response (Ferguson, 1986) but this remains to be proven. Given the "protective" reduction in thyroid hormone concentrations associated with NTI in dogs, it seems unlikely that any change in dosing strategy to maintain the relative concentrations of T_3 and T_4 is required in these cases. The mechanisms by which NTI and drug therapy can interfere with thyroid metabolism are reviewed in Chapter 8.

Ultimately, the pivotal determinant of the clinical effect of THRT is the molecular and cellular action of the hormones. As indicated above, the production of proteins, and maturation and growth of body tissues has clinical utility long after the thyroid hormones integral to their development have been metabolised and eliminated. The biological half lives of the hormones are therefore considerably longer than the corresponding circulating half lives.

Therapeutic Regimes

Choice of Product

Worldwide, a number of products for THRT are available. These can be divided into crude thyroid products, and synthetic preparations. The crude products are derived from desiccated porcine, ovine, or bovine thyroid glands. The thyroid hormone content in these products is more variable between preparations and between batches compared with synthetic hormone products (Rosychuk, 1982). Bioavailability is therefore not only greater but also more consistent for synthetic compared to crude preparations. The crude product shelf life is also shorter than that of the synthetic drugs. Within the UK, there are no licensed crude thyroid hormone products currently available for THRT in dogs and synthetic T_4 is the product of choice for almost all cases of canine hypothyroidism (Ferguson, 1986). The remainder of this review will therefore deal only with synthetic products which offer greater stability and standardisation of potency (Rosychuk, 1982). Various synthetic THRT preparations exist, consisting of T_4, T_3, or in some cases a combination of both hormones.

A number of synthetic T_3 products for THRT are available outside the UK. However, there are few indications for these products. As already stated, T_3 administration circumvents the normal physiologic process of T_4 deiodination to T_3. Consequently whilst circulating total and free T_3 concentrations may be within the therapeutic range after T_3 administration, total and free T_4 concentrations remain subnormal. The variable T_3 and T_4 requirement of various tissues also makes T_3 administration less desirable. Administration of purely T_3-containing products may result in adequate concentrations in organs such as the liver, kidneys and heart which derive their T_3 from the circulation. However, the principal source of intra-pituitary free T_3 is deiodination of free T_4 within the pituitary gland by type II 5'-D. Therefore, the brain and pituitary gland may be deficient in free T_3 if circulating concentrations of free T_4 are subnormal. Conversely, it has been suggested that administration of sufficient T_3 to provide adequate brain and pituitary concentrations may result in excessive concentrations in other organs (Ferguson, 1986). In addition, since the half life of T_3 is much shorter than that of T_4 more frequent dosing is required if circulating concentrations are to be maintained. One of the uncommon situations in which T_3 THRT may be of use, is in cases with small intestinal malabsorptive diseases. In these cases, the improved absorption characteristics of T_3 compared to T_4 has been suggested to be beneficial (Panciera, 1990b) but this remains to be proven.

Various commercial products containing a combination of T_4 and T_3 are available for use in THRT. The objective of the combined products is to mimic normal thyroidal secretion. However, most preparations contain a lower T_4 to T_3 ratio that that which is normally secreted in dogs (Laurberg, 1977). Administration of these products at the standard dose tends to result in low or low-normal circulating total T_4 concentrations in dogs. However, using an increased dose to normalise total T_4 usually results in excessive total T_3 concentrations, potentially capable of producing thyrotoxicosis (Ferguson, 1986).

The only licensed product in the UK for THRT in dogs is Levothyroxine Sodium, (**Soloxine**, Daniels Pharmaceuticals). This is a chemically derived compound which is identical to naturally occurring L-T_4. This product is the treatment of choice for several reasons. First, L-thyroxine is the principal secretory product of the thyroid gland and is the physiologic "pro-hormone" for the more potent T_3. Since re-establishment of normal circulating T_4 and T_3 concentrations is desirable, this is best achieved by administration of T_4. Pituitary synthesis and secretion of TSH is most effectively regulated by free T_4 concentrations. The improved standardisation and potency of the synthetic T_4 product compared with crude products is also preferable and reduces the risk of between-batch variability.

Choice of Dosing Regime

Whilst it is widely accepted to be the product of choice for treating hypothyroidism in dogs and humans, the dosing schedule for synthetic T_4 in dogs remains controversial. Since therapy is invariably lifelong, the identification of the optimal therapeutic and economic regime for an individual is important. In addition, a regime must be practical to implement chronically to ensure optimal owner compliance.

Kemppainen & Sartin, (1984) evaluated circulating total T_4 concentration every 20 minutes for 25 hours in nine normal beagle dogs exposed to continuous 12 hour light/dark cycles. The study confirmed episodic but not circadian release of T_4. Mean (± s.d.) total T_4 concentration was 17.8 (± 4.4) nmol/L with 3.3 (± 2.9) peaks per day. Consequently, there are no recommendations on the time of day most suitable for THRT which is likely to be of no importance as long as it remains reasonably consistent between days.

Most T_4 product supplementation protocols reported in the literature suggest using a total daily dose ranging from 0.02-0.04 mg/kg body weight. However recommendations have included giving this total dose either once, or divided twice or three times daily (Halliwell, 1979; Martin & Capen, 1979; Rosychuk, 1982). Alternative dosing schedules

have recommended dosing based on body surface area (0.5 mg/m^2 per day) (Chastain, 1982). The use of divided dose BID therapy results in less fluctuation of circulating T_4 concentrations compared to administration of the same total dose as a single daily bolus (Nachreiner & Refsal, 1992; Nachreiner *et al.,* 1993). However, as discussed above, the biologic action of the thyroid hormones exceeds that of the plasma half life making once daily therapy successful in most cases (Rosychuk, 1982; Ferguson, 1986; Panciera, 1997c). Whilst acknowledging this fact several authors do nevertheless recommend twice daily dosing schedules to maximise the therapeutic response, with some subsequently reducing the dose to once daily if the response is favourable (Stogdale, 1980; Panciera 1990b; 1997c).

Nachriner & Refsal, (1992) evaluated thyroid hormone results from 2674 dogs receiving THRT. In that study an increase in dose of greater than two fold caused a mean increase in peak total T_4 concentration of 59 %. The authors suggested that this may have resulted from less efficient absorption at the higher doses, saturation of binding proteins or dose dependent kinetics.

The dose requirement to achieve "therapeutic" blood hormone concentrations was investigated by Nachreiner *et al.,* (1993) who determined the pharmacokinetics of T_4 administered to thyroidectomised dogs. Doses varying from 0.011-0.044 mg/kg given once or twice daily were examined. Not surprisingly, peak circulating total T_4 concentrations were greater at the larger doses. In addition, the larger daily doses were also associated with a decrease in biologic half life of the hormone, supporting the concept of dose-dependent kinetics. Broadly speaking doubling the dose of T_4 administered tended to increase the peak circulating total T_4 concentration by approximately 60 %, supporting the results of the earlier study by Nachreiner & Refsal, (1992). Marked inter-individual variation in hormone pharmacokinetics was reported, which was generally consistent within dogs at each of the dosing regimens. Thus some individuals required higher doses or frequency of therapy to achieve similar blood total T_4 concentrations than did others. Therapy may therefore have to be highly individualised and tailored to the requirements of the individual dog.

Complications of Treatment

Most complications following the institution of THRT in hypothyroid dogs result from either inadequate or excessive dosage. Suboptimal dosing will simply result in either an

inadequate improvement, or no improvement at all. Biochemical confirmation of this can be easily achieved using the normal monitoring strategy described below. Although excessive THRT may result in signs of hyperthyroidism, dogs, unlike humans, are largely resistant to the effects of over-supplementation and only occasionally develop clinical signs of thyrotoxicosis (Kaptein *et al.,* 1994). This is probably due to the rapid metabolism of thyroid hormones in dogs, particularly biliary and faecal excretion. In addition, the considerable reserve binding capacity of the thyroid hormone binding proteins, is capable of compensating for marked increases in total thyroid hormone concentration without serious alterations in free hormone values (Larsson *et al.,* 1985). Since it is the free hormone that is most metabolically relevant, this further improves the dog's resistance to iatrogenic hyperthyroidism. Iatrogenic thyrotoxicosis is more likely when T_3 products are employed (Rosychuk, 1982; Panciera, 1997a) since the active hormone is faster acting and more metabolically potent than T_4.

Whilst some studies have required the use of up to 20 times the standard dose of THRT to induce clinical thyrotoxicosis in dogs (Piatnek & Olson, 1961), it is apparent that there is considerable inter-individual variation in susceptibility to thyrotoxicosis (Belshaw & Rijnberk, 1979; Panciera, 1990b). However, the reason for this remains unclear. The clinical signs of exogenous thyrotoxicosis include polydipsia, polyuria, polyphagia, panting, weight loss, tachycardia and pyrexia. A diagnosis can be confirmed by demonstration of grossly elevated circulating thyroid hormone concentrations, and signs should resolve within several days of stopping therapy (Jeffers, 1990).

Gradual introduction of THRT has been recommended in dogs with decreased ability to metabolise T_4 and increased risk of thyrotoxicosis such as hypoadrenocorticoid, aged, cardiac and diabetic patients. A divided dose protocol, starting with 25 % of the normal dose, increasing by 25 % increments every fortnight until optimal stabilisation is achieved is widely cited within the literature (Ferguson, 1986; Jeffers, 1990; Panciera, 1990b). However, these guidelines are largely empirical and whilst there is general agreement that care is needed in treating cases with concurrent hypothyroidism and hypoadrenocorticism (Rosychuk, 1982; Bowen, Schaer & Riley, 1986; Ferguson, 1986) there is relatively little evidence of problems associated with THRT in the presence of most other concurrent diseases in dogs (Rosychuk, 1982).

Therapeutic Monitoring

The evaluation of the success or failure of therapy in patients receiving THRT should be primarily a clinical judgment. Resolution of certain clinical signs such as mental dullness and lethargy may occur within as little as seven days after commencing therapy (Panciera, 1994), although others such as dermatological abnormalities may take up to five months to improve (Rosychuk, 1982; Ferguson, 1986). As normal follicle turnover is re-established after commencing THRT, there is frequently an increased shedding of hair prior to obvious regrowth. Consequently, worsening of the alopecia is not an uncommon finding in the initial stages of therapy (Rosychuk, 1982). There is also variation in the extent of clinical improvement reported with THRT depending on the system involved. Whilst metabolic and dermatological abnormalities improve very markedly, others including neurological abnormalities, megaoesophagus and laryngeal paralysis show a much poorer response to therapy (Panciera, 1994). This may be partly related to the dubious relationship of hypothyroidism in the aetiology of these abnormalities as discussed in Chapter 5, as well as a genuine differences in response between the various body systems. Rosychuk, (1982) suggested that weight loss occurs within the first four to six weeks, but is not completed for several months after starting THRT. Panciera, (1994) reported weight loss in the majority of 34 treated hypothyroid dogs with follow up information within eight weeks of starting THRT.

Clearly a valid evaluation of the response to THRT, particularly in a case with a suboptimal clinical response, can only be made if therapeutic circulating hormone concentrations are known, and therefore post-pill monitoring samples should be part of the monitoring process. It has been suggested that a "steady-state" condition is not achieved and therefore this initial monitoring should not be performed until approximately four to eight weeks after starting THRT, or after changing the dosing regime, in both humans and dogs (Rosychuk, 1982; Ferguson, 1986; Panciera, 1990b; Brent & Larsen, 1996; Refsal & Nachreiner, 1997b).

Nachreiner & Refsal, (1992) evaluated total and free iodothyronine concentrations in 2674 blood samples from dogs receiving THRT following removal of those cases with T_3Ab or T_4Ab. Whilst the study was laboratory based and evaluation of clinical response was not performed, the high number of samples analysed helped to compensate for this. Peak circulating concentrations of each of the four iodothyronine fractions occurred 4.1 to six hours after oral hormone administration irrespective of once versus twice daily therapeutic schedules. The peak concentrations were generally higher in dogs which

received once daily therapy compared to those in which the total daily dose was split. Dogs receiving a veterinary synthetic L-T_4 preparation had significantly greater circulating total and free T_4 concentrations compared with a commonly used human L-T_4 preparation, and various other generic preparations as a group. The authors suggested that the most likely explanation for this was variation in the thyroxine content between preparations. For equivalent doses, generic products typically resulted in peak thyroid hormone concentrations approximately 10 to 15 % lower than the proprietary products. Larger dogs tended to have lower therapeutic hormone concentrations for a given dose compared to smaller dogs. No definite explanation was provided for this finding, although Reimers *et al.,* (1990) demonstrated decreased naturally occurring thyroid hormone concentrations in large breed dogs compared with smaller breeds, and a normal physiological difference between breeds may therefore also be important in treated cases. There was marked inter-dog variation in the dose of thyroid hormone supplement required to achieve therapeutic hormone concentrations. Therefore failure to adequately respond to THRT may be the result of inadequate dosing for that individual, and confirmation of this requires appropriate blood hormone measurements.

In humans receiving THRT, objective therapeutic monitoring is principally achieved by circulating TSH estimation. Return of circulating TSH concentration to the reference range is generally associated with good clinical control. Human TSH assays are capable of differentiating "normal" results from the subnormal values typical of over supplementation (Brent & Larsen, 1996). However, the currently available commercial cTSH assays are unable to differentiate normal from subnormal values, therefore reducing the utility of this measurement for therapeutic monitoring in dogs (Refsal & Nachreiner, 1997b). The precise serum thyroid hormone profile associated with good clinical response has not been identified in dogs with spontaneous hypothyroidism (Refsal & Nachreiner, 1997b). However, in one report, treatment of experimentally induced hypothyroidism in beagles was accomplished with a mean dose of 0.034 mg/kg/day (Hesch and Koehrle, 1986). It has been suggested that peak concentrations in the normal to high-normal range, are a reasonable objective (Ferguson, 1986; Panciera, 1990b). Refsal & Nachreiner, (1997b) suggested that elevated peak circulating concentrations may be a desirable objective end-point of therapy. No "maximum" hormone concentration whereby thyrotoxicosis is probable in dogs has been identified in clinical studies, although experimental studies using massive doses have certainly achieved this (Piatnek & Olson, 1961). The absence of this clinical data may partly reflect the capacity of dogs to compensate for over-

supplementation of THRT at most commonly used dosing regimes. In dogs with increased peak total T_4 concentrations, total T_3 results tend to be within the reference range indicating physiological adaptation to avoid thyrotoxicosis (Nachreiner & Refsal, 1992). Pre-pill "trough" concentration measurement has also been advocated as a possible monitoring tool (Feldman & Nelson, 1996), and Ferguson, (1986) suggested that given the dog's resistance to the development of thyrotoxicosis, nadir thyroid hormone concentrations in the "normal" range may be desirable.

The return of routine clinicopathological parameters to within the appropriate reference limits can be used to support the interpretation of an individual's clinical response and circulating thyroid hormone concentrations during therapeutic monitoring (Feldman & Nelson, 1996). However whilst Feldman & Nelson, (1996) suggested that improvements in routine clinical pathology occur within two to four weeks of starting THRT in dogs, very little data have been published.

6.2 INTRODUCTION

Many protocols for the treatment and therapeutic monitoring of canine hypothyroidism have been advocated. However, whilst general guidelines are provided in the veterinary literature, there is very little objective information with which to correlate the clinical and clinicopathological response to therapy in dogs. In particular, being a fairly novel test, there is limited information with which to evaluate the role of cTSH estimation in therapeutic monitoring. In addition, the most appropriate time after starting THRT before monitoring should be performed is unclear. Similarly there is a dearth of data concerning the detail of clinicopathological changes in hypothyroid dogs following the start of THRT. The objectives of this study were to evaluate the clinical response to therapy using a commonly used dosing schedule, and investigate in detail the endocrine and routine clinicopathological changes which occur during THRT in a group of dogs with reliably confirmed hypothyroidism.

6.3 MATERIALS AND METHODS

Case Material and Data Manipulation

Data from the 52 hypothyroid dogs discussed in previous chapters were initially considered for inclusion in the study. The clinical and clinicopathological parameters detailed in Chapter 2 were recorded for each case at each monitoring check. The data were grouped primarily according to case number and secondarily according to length of time on THRT. Duration of THRT was divided into four time frames, or "visits". This was performed to allow analysis of any change in clinical and clinicopathological parameters with increasing duration of THRT. The temporal criteria used to determine visits were chosen to provide a reasonable spread of times applicable in practice, with similar numbers of cases in each category. These data are summarised in Table 11.

In individual dogs that were seen on multiple occasions within an individual "visit" time period, the monitoring check with most recorded data was used and the other was excluded from the analyses. This prevented data from individuals biasing the mean results of that "visit" as a whole. The data recorded for each dog-visit are detailed in Appendix 4. Only cases with complete pre-treatment data and data from at least one follow up examination were included. To be included, data must have been recorded for duration of THRT, comments on clinical response, and circulating total T_4 and cTSH concentrations (collected six hours after tablet administration in those dogs receiving therapy). If any of

these data were missing, the dog-visit was automatically excluded. The great majority of dog-visits which were included also had results of body weight measurement, routine biochemistry and haematology analyses.

Visit	Duration of THRT (days)
1	Pre-treatment
2	9-28
3	29-70
4	71-112

Table 11. Temporal criteria used to define each of the "visit" periods into four distinct groups based on the length of time on thyroid hormone replacement therapy (THRT).

As a result of the strict criteria used for inclusion, 21 cases were excluded because there were insufficient repeated analyses with adequate data following the start of THRT. These typically occurred due to owners' financial restraints or more commonly failure to regularly return for follow up examinations. The approximate follow up times requested of owners at the outset of the study were two, six and 12 weeks after the start of THRT. These periods were subsequently modified in individual cases as appropriate depending on factors such as response to therapy or the development of concurrent problems. Body weight data were calculated and analysed as percentages of the animals pre-treatment weight. This was performed to eliminate the biasing effect that would be exerted by particularly large or small dogs in subsequent analyses, since not all dogs had body weight data for every visit.

Treatment Protocol

All dogs were started on synthetic T_4 replacement therapy as described in Chapter 2. Thyroid hormone doses approximating 0.02 mg/kg given once daily were used initially and subsequently modified as clinically required.

Therapeutic Monitoring

Samples were collected for total T_4, cTSH and routine biochemical and haematological profiles six hours after T_4 administration. Sample handing and analyses were performed as described in Chapter 2. A full clinical examination was performed at each visit and changes in clinical signs including body weight were recorded as described in Chapter 2.

Statistical Analysis

Data were sorted and stored as described in Chapter 2. The effect of time period on clinicopathological parameters was evaluated using a general linear model analysis of variance with post hoc pairwise comparison of means performed by Newman-Keuls multiple range testing. All statistical computations were performed using MINITAB 9.2 for Windows (Minitab Inc.). Clinicopathological results were categorised into those from cases who needed an increase in THRT dosage at any point during the study and those in which the dosage was not changed. Comparison of the clinicopathological parameters at visit two between these two groups was performed using a Mann-Whitney U test.

6.4 RESULTS

The number of dog-visits included in each visit group, and the associated temporal data are detailed in Table 12. The mean results of all endocrine analyses categorised by visit are detailed in Table 13. Median total T_4 concentrations were 4.7, 52.0, 46.7 and 52.3 nmol/L at visits one to four, respectively. The total T_4 results had a relatively gaussian distribution when plotted and manually inspected. This was supported by similar mean and median results. However, examination of the cTSH results revealed a considerable bias in distribution with median results of 0.03, 0.03 and 0.02 ng/ml at visits two, three and four, respectively. A small number of cases with particularly increased results were responsible for this effect. Circulating cTSH results were above the reference range in 26 of 31 (83.9 %), two of 26 (7.7 %), one of 25 (4 %) and one of 22 (4.5 %) cases at visits one, two, three and four, respectively. Clinical and endocrine results from each dog at each visit are detailed in Appendix 33.

	Dogs with complete data	Days on treatment mean	s.d.	min	max
Visit 1	31	NA	NA	NA	NA
Visit 2	26	17.77	4.85	9	28
Visit 3	25	49.36	10.89	29	69
Visit 4	22	95.91	11.25	77	112

Table 12. Summary figures of the number of dogs included in each visit period, and the corresponding group temporal data for duration of thyroid hormone replacement therapy.
NA Not Applicable

	Mean ± s.d. (range) total T_4 (nmol/L)	Mean ± s.d. (range) cTSH (ng/ml)
Visit 1 (pre treatment)	7.0 ± 3.9 (4.7-17.8)[2,3,4]	2.51 ± 2.68 (0.01-12.3)[2,3,4]
Visit 2	54.7 ± 24.8 (8.3-100.5)[1]	0.17 ± 0.35 (0.01-1.65)[1]
Visit 3	51.8 ± 18.2 (26.8-103.5)[1]	0.16 ± 0.33 (0.01-1.62)[1]
Visit 4	56.5 ± 22.9 (12.6-116.5)[1]	0.12 ± 0.23 (0.01-1.00)[1]

Table 13. Total T_4 and cTSH concentrations obtained from dogs receiving thyroid hormone replacement therapy categorised by visit. All samples from were collected six hours after T_4 administration.

[1] significantly different from visit one
[2] significantly different from visit two
[3] significantly different from visit three
[4] significantly different from visit four

The dosing adjustments and mean dose concentrations (per kg body weight) grouped by visit period are summarised in Table 14. Of the 31 dogs on treatment, dose adjustments were required in 15 of them at some point during the course of therapy. The dose of THRT was increased in 11 dogs (five dogs at visit two, four dogs at visit three and two dogs at visit four) and decreased in four dogs (three dogs at visit three and one dog at visit four) as outlined in Table 14. The mean dose of T_4 administered to all dogs was 0.0200, 0.0210, 0.0248 and 0.0284 mg/kg body weight at visits one, two, three and four, respectively. The cases in which a dose change was required are detailed in Table 15. The mean dose of T_4 administered to the dogs which did not require a change in dose at any

time during the study was 0.0201, 0.0213, 0.0211 and 0.0227 mg/kg body weight at visits one, two, three and four, respectively. In one dog with chronic diarrhoea, an unusually large dose of T_4 replacement therapy was required (0.076 mg/kg) to obtain an adequate clinical response and adequate six hour post-pill total T_4 concentration. Twice daily therapy was not considered necessary in any of the 31 dogs.

	Visit 1	Visit 2	Visit 3	Visit 4
Mean dose of therapy (mg/kg)	0.0200	0.0210	0.0248	0.0284
Dogs needing increased dose	NA	5	4	2
Dogs needing decreases dose	NA	0	3	1

Table 14. Dosing schedules and number of cases requiring a change in dose of therapy at each visit out of 31 hypothyroid dogs receiving thyroid hormone replacement therapy.

Dog Number	Hospital Number	Visit	Total T_4 (nmol/L)	cTSH (ng/ml)	Direction of adjustment	Dose change (mg)
2	131136	3	38.4	0.03	Increase	0.5 to 0.8
3	131132	2	13.3	1.65	Increase	0.2 to 0.3
4	131486	3	26.8	0.54	Increase	0.15 to 0.3
5	129827	3	43.5	0.01	Decrease	0.5 to 0.3
6	133137	4	23.0	4.00	Increase	0.5 to 0.7
10	132396	3	103.5	0.01	Decrease	0.6 to 0.5
11	122647	2	42.3	0.14	Increase	0.6 to 0.8
13	130375	3	49.2	0.39	Decrease	0.3 to 0.15
14	134156	4	31.4	0.15	Increase	0.5 to 0.8
21	127111	4	95.9	0.19	Decrease	0.7 to 0.5
22	133778	3	39.8	0.01	Increase	0.5 to 0.6
23	133641	2	29.3	0.75	Increase	0.5 to 0.8
24	132391	2	8.3	0.02	Increase	0.6 to 2.0
25	127627	2	29.1	0.01	Increase	0.3 to 0.4
30	129757	3	31.4	0.22	Increase	0.8 to 1.0

Table 15. Summary of cases requiring alteration in thyroid hormone replacement therapy.

Two dogs with subnormal peak total T_4 concentrations required an increase in dosage at visit two based on an inadequate clinical response to therapy. One (dog 24) had a peak total T_4 concentration of 8.3 nmol/L. There was persistent diarrhoea in this case and poor gastrointestinal absorption was suspected as the reason for initial therapeutic failure. Following an increase in dose subsequent peak total T_4 results (41.9 and 40.9 nmol/L) were consistent with adequate absorption and this was matched by resolution of clinical signs. In the remaining dog (dog 3), the peak total T_4 result was subnormal (13.3 nmol/L). In the other cases that required an increased dose, peak total T_4 values were within the reference range but the clinical response was considered suboptimal. In one of these cases (dog 6), the dosage change was made between visits three and four and therefore these data are not shown in Appendix 33. The relevant monitoring total T_4 and cTSH values in this case when the dosage adjustment was made were 23.0 nmol/L and 4.0 ng/ml, respectively. For statistical purposes, these data were assigned to visit four. Although many of the dogs which needed an increase in therapy had reference range peak total T_4 concentrations, the results were generally lower than those in which an increase in dose was not required. Eight of the 11 dogs requiring an increase in dosage had peak total T_4 values < 35nmol/L. In contrast, the minimum peak total T_4 concentration obtained at any visit from all the dogs in which no dosage increase was required was < 35 nmol/L on only one occasion.

Four of the 31 dogs required a decrease in therapy. In two of these dogs (dogs 10 and 21) peak total T_4 results were approximately 100 nmol/L although only mild clinical signs of hyperthyroidism (polydipsia and hyperactivity) was present in just one of them. Two additional cases (dogs 6 and 9) had peak total T_4 concentrations approximately 100 nmol/L but neither of these cases required a reduction in therapy. Following the reduction in dose in dog 10, a subnormal peak total T_4 concentration (12.6 nmol/L) was obtained from a subsequent monitoring sample. However, this was attributed to failure of appropriate therapy on the day of testing since the dog was clinically well controlled and the corresponding cTSH result (0.01 ng/ml) was at the assay LoD. The dose of therapy did not subsequently need to be adjusted in this case. In the other two cases requiring a reduction in dosage (dogs 5 and 13), peak total T_4 results were not excessive (43.5 and 49.2 nmol/L) but clinical signs of hyperthyroidism were apparently present (marked hyperactivity in both cases). The reduction in dose in these two dogs was associated with an improvement in the unwanted signs. However, it was difficult to ascertain if the dogs were genuinely clinically hyperthyroid or if the behavioural changes considered undesirable

by the owners were only a relative change compared to the pre-treatment demeanour. In two of the four dogs requiring a dose reduction, cTSH was equal to the assay LoD.

At each treatment visit, most dogs had circulating cTSH concentrations near the assay LoD. Seven of the dogs which needed an increase in dosage had cTSH values greater than the median value for all dogs at the corresponding visit. In the remaining four cases, the cTSH result was approximately the median group value for the visit but an increase in dose was clinically required. Circulating cTSH values were similar to the visit median in two of the four dogs (dogs 5 and 10) in which a decrease in therapy was required, but greater than the median result in the remaining two cases (dogs 13 and 21).

The relative changes in body weight categorised by visit are detailed in Table 16.

	Percent pre-treatment body weight
Visit 1	100 [2,3,4]
Visit 2	95.8 ± 3.9 [1,4]
Visit 3	93.2 ± 4.8 [1,4]
Visit 4	91.3 ± 5.5 [1,2,3]

Table 16. Change in body weight following therapy for hypothyroidism. All values are expressed as mean (± s.d.) percentage of the original (pre-treatment) body weight.

[1] significantly different from visit one
[2] significantly different from visit two
[3] significantly different from visit three
[4] significantly different from visit four

Broadly speaking all dogs exhibited either a good or excellent clinical response to THRT. Some clinical improvement was noticed in almost all cases by visit two. Metabolic signs such as mental demeanour and willingness to exercise were usually the first signs to improve and were typically reported to start within three or four days of therapy. Approximately half of all owners spontaneously commented that the dog was behaving "more like a puppy". Hair coat improvements were normally reported by visit three. Commonly there was a period of initial hair loss prior to re-growth. In a number of cases, the hair coat was considered normal by visit three, although whilst improved, many dogs still had some hair coat abnormalities by visit four. The most obvious dermatological

improvements were regrowth of hair, and resolution of pyoderma. In several cases there was darkening of the hair coat. In one case, the owner commented that the dog had started barking again, although the loss of vocation had not been identified as a problem prior to starting therapy. There was nearly universal improvement in the level of activity which was exhibited by the treated dogs, including those cases in whom this was not recognised as a problem before starting therapy. In one dog with corneal lipidosis, the ocular abnormalities did not resolve with therapy and in fact continued to deteriorate despite normalisation of the dog's lipid profile. In other respects this dog clinically improved. One dog developed an acute temporary lameness during THRT but this was not considered related to the treatment and responded to alternative therapy. Two dogs with concurrent diabetes mellitus, and one dog with concurrent dilated cardiomyopathy were treated with the same dosing protocol of THRT as the other cases. None of these three cases needed a change in dose strategy, and no worsening of the concurrent problems were identified as a result of instituting THRT.

The biochemical and haematological results from each dog at each visit are detailed in Appendices 34 and 35. The results of routine biochemical analyses categorised by time period are detailed in Table 17. The results of routine haematological analyses categorised by time period are detailed in Table 18. Significant changes in results of sodium, calcium, phosphate, glucose, cholesterol, creatinine, ALKP, AST, total protein, albumin, globulin, triglycerides, fructosamine, RBC count, haemoglobin, MCV and MCH occurred at various times after the start of THRT. These changes are detailed in the relevant Tables. Mean (± s.d.) circulating creatinine concentration was significantly ($p<0.01$) greater at visit two in those dogs which needed an increase in THRT dosage at some point during the study (107 ± 24.1 μmol/L) compared to those in which no adjustment was necessary (77.1 ± 16.7 μmol/L). No other routine parameters were significantly different between these two groups.

	Visit 1	Visit 2	Visit 3	Visit 4	P-value
Urea	4.7 (3.1)	5.1 (2.5)	4.3 (2.7)	4.6 (2.4)	0.463
Sodium	144.0 (3.0)[2,3,4]	147.0 (3.0)[1]	146 (2.2)[1]	147 (3.0)[1]	0.000
Potassium	4.6 (0.7)	4.4 (0.6)	4.4 (0.47)	4.30 (0.72)	0.457
Chloride	110.5 (8.5)	112.0 (7.0)	114.0 (4.0)	113.0 (2.5)	0.093
Calcium	2.68 (0.27)[4]	2.65 (0.27)[4]	2.61 (0.24)[4]	2.52 (0.23)[1,2,3]	0.007
Magnesium	0.80 (0.18)	0.74 (0.23)	0.76 (0.14)	0.73 (0.15)	0.197
Phosphate*	1.26 (0.29)	1.40 (0.29)	1.43 (0.44)	1.34 (0.25)	0.046
Glucose	5.45 (0.78)[3]	5.35 (0.97)	5.50 (1.05)[1]	5.55 (0.90)	0.025
Cholesterol	10.51 (6.25)[2,3,4]	5.63 (2.15)[1]	4.60 (2.73)[1]	4.68 (2.46)[1]	0.000
Creatinine	105.0 (39.0)[2,3,4]	94.0 (46.0)[1]	93.0 (39.2)[1]	93.5 (28.2)[1]	0.000
Bilirubin	1.0 (1.0)	0.0 (0.0)	1.0 (0.75)	1.0 (0.0)	0.258
ALKP	138 (305)[3,4]	126 (181)	105 (102)[1]	117.0 (113)[1]	0.019
AST	24.5 (15.2)[3,4]	25.0 (12.0)[4]	21.5 (9.0)[1]	19.0 (7.7)[1,2]	0.012
ALT	43.5 (73.3)	52.0 (171)	42.5 (50.4)	24.5 (28.5)	0.074
Total Protein	66.5 (10.2)[3,4]	64.0 (13.0)	63.0 (8.7)[1]	62.0 (11.7)[1]	0.015
Albumin	35.0 (7.0)[4]	37.0 (7.0)[4]	35.0 (8.0)	32.5 (5.7)[1,2]	0.034
Globulin	31.5 (6.2)[4]	28.0 (5.0)[4]	28.0 (4.5)	29.0 (7.25)[1,2]	0.021
Creatine Kinase	100 (86)	117 (97)	114 (62)	104 (67.5)	0.845
Triglycerides	1.56 (4.05)[2,3,4]	1.18 (0.81)[1]	0.90 (0.78)[1]	0.97 (0.96)[1]	0.003
γ-GT	5.0 (4.0)	3.0 (2.0)	4.0 (2.0)	4.0 (1.2)	0.463
Fructosamine	305 (77)[2,3,4]	297 (53)[1,3,4]	250 (77)[1,2,4]	251 (80)[1,2,3]	0.000

Table 17. Changes in routine blood biochemical parameters (median (semi-interquartile range)) in hypothyroid dogs following treatment with thyroid hormone replacement therapy, and the associated p-value for the overall effect of treatment.

[1] significantly different from visit one [2] significantly different from visit two

[3] significantly different from visit three [4] significantly different from visit four

* overall effect of therapy significant ($p<0.05$) but no individual time points significant

	Visit 1	Visit 2	Visit 3	Visit 4	P-value
RBC count	5.57 (1.22)[2,3,4]	5.96 (0.97)[1,3,4]	6.30 (1.00)[1,2,4]	6.72 (0.72)[1,2,3]	0.000
Haemoglobin	12.9 (2.4)[2,3,4]	13.5 (2.2)[1,3,4]	14.3 (1.6)[1,2]	15.1 (1.9)[1,2]	0.000
MCV	66.0 (5.0)[4]	68.0 (5.5)[4]	66.0 (6.5)[4]	63.5 (5.0)[1,2,3]	0.000
MCH	23.3 (1.9)[4]	23.7 (1.4)[3,4]	23.2 (1.7)[2,4]	22.6 (1.7)[1,2,3]	0.000
MCHC	34.5 (1.3)	34.6 (1.6)	34.3 (2.2)	34.9 (1.6)	0.740
Platelets	365 (217)	381 (224)	322 (195)	276 (196)	0.146
MPV	8.3 (1.3)	8.8 (1.8)	8.8 (1.3)	8.95 (1.5)	0.165
WBC	9.5 (4.7)	11.0 (5.3)	12.1 (6.8)	10.7 (4.6)	0.767
Band N	0.0 (0.09)	0.0 (0.06)	0.00 (0.12)	0.0 (0.22)	0.947
Neutrophils	6.7 (4.1)	7.9 (3.5)	8.1 (6.2)	7.9 (4.8)	0.799
Lymphocytes	1.8 (0.8)	1.7 (1.2)	1.9 (1.2)	2.2 (1.0)	0.245
Monocytes	0.57 (0.55)	0.42 (0.61)	0.38 (0.47)	0.47 (0.31)	0.661
Eosinophils	0.33 (0.36)	0.26 (0.38)	0.41 (0.37)	0.34 (0.43)	0.691

Table 18. Changes in routine haematological parameters (median (semi-interquartile range)) in hypothyroid dogs following treatment with thyroid hormone replacement therapy, and the associated p-value for the overall effect of treatment.

[1] significantly different from visit one [2] significantly different from visit two

[3] significantly different from visit three [4] significantly different from visit four

6.5 DISCUSSION

In this study, 16 of the 31 dogs did not need any adjustment in the dose of THRT following the start of treatment. An increase in dose was required in 11 of the dogs, whilst four dogs needed a dose reduction. This supports the presence of individual variation in the requirement for THRT previously reported in experimental cases by Nachreiner *et al.*, (1993). Broadly speaking, a peak total T_4 concentration of less than 35 nmol/L was common in dogs which needed an increase in dosage, but was rare in dogs who were clinically adequately treated. This value may therefore be used as a guide to the likelihood of clinical response to therapy. No dogs needed more than one dose adjustment. The time required after starting therapy until a dose adjustment was required was variable. However, most dose alterations were typically required at one of the first two checks. Only three of 31 (9.7 %) dogs required a dosage change at visit four. Overall, there was a slow increase in the mean (per kg) dose of T_4 required to maintain good clinical control from a starting dose of 0.0200 mg/kg/day to a final mean dose of 0.0284 mg/kg/day. This may partly reflect the reduction in mean body weight of the dogs after starting treatment, artificially increasing the mean daily dose. However, this was not solely responsible, since the mean body weight of all the dogs as a group decreased by less than 10 % during the study period, whereas the mean dose of THRT increased by over 40 % during this time. Of those dogs which did not require a change in dose, the mean dose of therapy per kg body weight increased broadly in line with the mean reduction in body weight. This supports the finding that in certain individuals, a genuine requirement for an increase in therapy to achieve optimal clinical control exists. However, clearly this is not the case in all dogs, again demonstrating the variation in response of dogs to THRT.

The measurement of cTSH in dogs receiving THRT demonstrated that there is a rapid and profound decrease in circulating cTSH in most dogs within approximately two weeks of commencing therapy. No assessment of cTSH was made prior to this time, and therefore the exact time taken for the suppressive effect of therapy to alter cTSH values, remains unclear. The results demonstrated that return of cTSH to the euthyroid range occurs in the majority of treated dogs and in most cases, results are usually at or near the assay LoD. However, at each visit there were a number of dogs with persistently increased cTSH values. In most cases (dogs 3, 6, 23) as detailed in Table 15 and Appendix 33, these corresponded to dogs with a clinical requirement for increased therapy. The monitoring cTSH concentration was increased in only one dog (dog 11, visit 3) in which no increase in therapy was clinically required at that time. This dog had developed a temporary unrelated

musculoskeletal condition when the inappropriately increased cTSH result was identified. No change in THRT was made at that time and none was subsequently required. It is possible that the dog was recovering from the concurrent illness at the time of testing. With this exception, the results indicate that cTSH within the hypothyroid range is usually indicative of a clinical requirement for an increase in THRT dosage. However, in many of the dogs (dogs 2, 4, 11, 14, 22, 24, 25 and 30) requiring an increase in therapy, the cTSH value was within the euthyroid range. Therefore, the cTSH result was of little use in confirming the adequacy of therapy. Using near complete suppression of cTSH as an indicator of the adequacy of therapy was not particularly useful either since four of 11 dogs with a clinical requirement for an increased dose had cTSH results at or near the assay LoD. Similarly, over-supplementation could not be reliably identified by demonstration of markedly suppressed cTSH values since two of four dogs in which the dosage was decreased had typical euthyroid range concentrations. The reason for the poor reliability of cTSH to identify dogs with a requirement for alterations in dose is unclear but may be related to the duration of therapy, owner compliance or individual case variation. This is certainly an area worthy of further study.

One limitation of the study is that the decision to make dose adjustments was based on consideration of clinical and clinicopathological results, including hormone concentrations. Potentially, this may have artificially altered the mean peak total T_4 concentration and mean dose of THRT per kg body weight. Nevertheless, the slow increase in dose of THRT is likely to be in part at least, due to a genuine physiological requirement since the overriding factor used to determine if dose adjustments were instituted was the clinical presentation and 11 of the 31 (35.5 %) dogs required an increased dose. Ultimately all dogs were well clinically controlled on once daily therapy, confirming that this therapeutic approach is appropriate in the vast majority of dogs. Once daily dosing is less physiologically normal than twice daily therapy, but the clinical response is clearly adequate and the cost of therapy is obviously reduced. In addition, the likelihood of improved owner compliance also makes this approach preferable.

There was biochemical or clinical evidence of hyperthyroidism in four of 31 (12.9 %) of cases. However, both biochemical and clinical evidence of this complication was only present in one dog, and the signs were mild. The resistance of at least some of the dogs to clinical hyperthyroidism despite markedly increased total T_4 concentrations supports previous reports (Kaptein *et al.,* 1994). However, it is unclear why the presence of hyperthyroid signs apparently occurred in two dogs with "high normal" peak total T_4

concentrations. The reduced dose requirement in these dogs was largely owner driven, and whilst the dogs may well have been "hyperexcitable", it is difficult to confirm that this was pathological and not simply a nuisance.

Several dogs with concurrent NTI including diabetes mellitus and DCM were treated with a standard therapeutic regime. No complications of this approach were recognised indicating that the widespread belief that such dogs should have THRT gradually introduced may not necessarily be the case (Ferguson, 1986; Jeffers, 1990; Panciera, 1990b). Confirmation of this would require study of a larger number of dogs with reliably confirmed hypothyroidism and concurrent NTI. One dog with chronic diarrhoea required a markedly higher dose of THRT to control the clinical signs. Despite eventual resolution of the diarrhoea, the peak total T_4 concentrations remained within the mid-normal range and clinical signs of hyperthyroidism did not develop, and so the dosing schedule was not modified. The findings in this dog are interesting. The high dosage requirement was initially presumed to be solely the result of the gastrointestinal disease. However, following successful treatment of the intestinal disease, typically "normal" six hour post pill total T_4 concentrations were obtained from this dog despite the continued use of an unusually high dose of THRT. The presence of residual intestinal pathology, capable of interfering with drug absorption, but not sufficiently severe to cause continued diarrhoea is an one possible explanation. Alternatively, the high dosage may reflect the marked inter-individual variation in requirement for THRT. However, perhaps the most likely explanation for the findings in this dog simply relate to the ability of this species to efficiently eliminate excess T_4 as discussed previously. An alternative initial approach to this case, when reduced intestinal absorption was suspected, would have been administration of a T_3 containing product (Panciera, 1990b). However, this was not deemed necessary.

The change in body weight of hypothyroid dogs following THRT was dramatic with significant reduction occurring by the first visit (mean duration 17.8 days) after commencing THRT. These results indicate that a standard dose of T_4 replacement therapy has a more rapid influence on body weight than was previously realised (Rosychuk, 1982; Panciera, 1997c). Interestingly, this pattern of weight loss continued throughout the period of study such that although no dogs suffered excessive weight loss, the mean body weight was significantly decreased at visit four compared with visit three. This confirms that the metabolic normalisation which results from THRT continues up until and possibly even beyond the mean visit-four period of 96 days after starting THRT.

Broadly speaking the changes in clinical signs which occurred following the start of THRT were similar to previous reports (Rosychuk, 1982; Jeffers, 1990; Panciera, 1997c). The changes in clinical parameters which could be objectively measured such as body weight have already been discussed. However the mean time required for most types of clinical abnormality to improve was difficult to accurately categorise. This is a common problem encountered in performing a clinical study such as this, since clinical improvements are usually progressive in nature. Furthermore variation between cases, such as in this study the need for a dose change, unavoidably occurs, complicating categorisation. Nevertheless, general conclusions can be drawn and whilst the time taken for improvement of clinical signs varied, it did undoubtedly depend on the body system affected. Lethargy was almost without exception improved by visit two and as can be seen from Table 15, weight reduction occurred quickly after starting therapy. Exercise tolerance appeared to improve continuously throughout the study as dogs became stronger and apparently more athletic. The reduction in body weight probably also contributed to this finding. Few dogs exhibited any change in hair coat by visit two, although this was usually improved by visit three. The deterioration and subsequent improvement in alopecia tended to occur during this interval. Although not specifically detailed, a number of the cases were intermittently checked for over a year beyond the initial study. Of these cases, no substantial clinical problems or complications were noted, although intermittent pyodermas were occasionally reported.

Circulating cTSH concentrations were significantly decreased at all visits compared to pre-treatment values, but were not significantly different between the treatment visits. Circulating cTSH concentration was within the reference range in 92.3 % cases by visit two compared with 16.1 % cases before treatment. It is clear from this that the negative feedback effect of THRT on pituitary cTSH release generally occurs rapidly after starting therapy. There was no significant change in mean six-hour post pill total T_4 concentration in monitoring samples between visits two, three or four. Whilst this is broadly consistent with the rapid stabilisation of thyroid hormone concentrations in most dogs, these data are influenced by the fact that dose alterations were made partly based on peak total T_4 results. As discussed previously, this will have contributed to maintaining similar mean peak total T_4 values between visits. Nevertheless, the recognition that cTSH and peak total T_4 are usually within the reference ranges within approximately two weeks after the start of therapy suggests that THRT can be adequately monitored much sooner than previously realised (Rosychuk, 1982; Ferguson, 1986; Panciera, 1990b; Brent & Larsen, 1996; Refsal & Nachreiner, 1997b). This has obvious beneficial effects for the welfare of treated dogs,

particularly those who require dosage changes to achieve an optimal clinical response. Based on the results of this study, approximately 20 % of cases may need a dosage adjustment at the first check after starting treatment, and approximately 30 % of dogs may need dosage adjustments at a subsequent visit. It is apparently rare for an individual to require more than one dose adjustment during the first three months of therapy.

The circulating total T_4 concentrations obtained at visits two, three and four are similar to or slightly higher than previous recommendations for therapeutic hormone concentrations (Rosychuk, 1982; Panciera, 1994; Refsal & Nachreiner, 1997b). These values, and the confirmation of minimal subsequent changes in either mean total T_4 or cTSH values after the initial visit, provides a guideline for objective clinicopathological monitoring results which may be used in future studies.

The effect of THRT on routine biochemical and haematological parameters produced a large amount of data to support previous anecdotal evidence in the veterinary literature. In addition it revealed a number of previously unreported features.

Circulating cholesterol and triglycerides were highly significantly decreased at visits two, three and four compared to the pre-treatment visit. There was no significant change in either cholesterol or triglyceride concentrations between the three treatment visits. This indicates that lipid metabolism is rapidly restored following the start of THRT, and treated cases demonstrate a quick normalisation of their lipid profile. This feature may be useful when monitoring dogs receiving THRT, particularly since these tests are easily performed and relatively inexpensive. However, there was no significant difference in the magnitude of the decrease in cholesterol or triglyceride concentrations at visit two in those dogs which were adequately treated, compared to those in which a subsequent dose adjustment was required. Therefore, whilst measurement of these parameters may assist in confirming that owner compliance is broadly acceptable, and that the dog is biochemically improving, the diagnostic utility of these analytes is of limited value in accurate therapeutic monitoring. Further study, with a larger number of samples from dogs needing dosage adjustments and from those not requiring a dose adjustment would be useful, since the relatively small sample numbers in this study may have reduced the likelihood of identifying any such statistically significant effects in the present study.

Blood glucose concentration was significantly decreased at visit three compared with visit one, but not between any other time periods. There is no obvious explanation for this finding. However, the median visit two blood glucose concentration was in fact even further decreased but was not statistically significant and therefore it is almost certain that

the statistical effect resulted from the number of samples in each of the groupings. The finding was considered to be a purely statistical and not biological effect. The result is therefore not considered to have any clinical relevance.

Circulating total protein concentration was significantly decreased at visits three and four compared to visit one. Albumin was significantly decreased at visit four compared to both visits one and two. The globulin concentration was significantly decreased at visits two and four compared to visit one. The most likely explanation for the protein concentration and profile changes which occurred is an increase in protein turnover associated with normalisation of metabolic rate, including energy consumption, following the start of THRT. There was a short lag phase before significant changes in total protein results were identified. This is consistent with a progressive metabolic improvement with THRT. The half life of albumin in dogs is over eight days in healthy dogs (Kaneko, 1980) and likely therefore to be even greater in hypothyroid animals, whose metabolic requirements and catabolic activity are reduced.

There was a marked, and highly significant reduction in mean circulating fructosamine concentrations following the start of THRT. This has not been previously reported in dogs. In humans with hypothyroidism, increased fructosamine concentration is recognised and considered to be due to reduced protein turnover rate rather than any abnormality of glycaemic control itself (Waterson & Mills, 1988). A similar mechanism is likely to be responsible in dogs, and the changes following instigation of THRT are presumably simply a reversal of this process. This conclusion is supported by the change in protein concentrations which occurred. The decrease in fructosamine concentration was statistically significant between every visit including visits three and four, indicating that even by a mean of 96 days of THRT, fructosamine may not have completely stabilised.

Mean circulating sodium concentration was significantly increased at all visits after starting therapy. However, the actual concentrations remained well within acceptable reference limits and were of no clinical significance. The cause of the change was likely to be a resolution of sample lipaemia, which artefactually decreases measured blood sodium concentration (Harrington & Cohen, 1973), after starting THRT.

Blood calcium concentration was significantly decreased at visit four compared to visits one, two and three. The concentration remained within acceptable reference limits and therefore was also of little clinical utility. The likely explanation for this previously unreported finding is an apparent decrease in calcium due to reduced circulating albumin

concentration which also occurred. Since total as opposed to ionised calcium was measured, the effect of protein fluctuations is unavoidable with the methodology used.

There was a highly significant reduction in creatinine concentration following the start of THRT, with values being reduced at each of visits two, three and four when compared to pre-treatment values, but not when compared to each other. This is a previously unreported finding and may be the result of improved glomerular filtration rate, as systolic blood pressure returns to normal. The creatinine production rate is directly proportional to lean muscle mass, and consequently to body weight. However, the reduction in body weight in the dogs in the present study is unlikely to have influenced creatinine concentration since most of the excess weight associated with hypothyroidism results from excess adipose tissue as opposed to muscle. The rapid change in creatinine concentration indicates that these metabolic improvements occur quickly following the start of replacement therapy. Mean body weight continually decreased throughout the study whereas mean creatinine concentration was not significantly different between visits two, three and four. Again, this indicates that the alteration in body weight was of little relevance to the changes in creatinine concentration.

Blood ALKP concentration was significantly decreased at visits three and four compared to visit one. Again, to the authors knowledge this is a previously unreported finding in dogs. Possible explanations include the progressive normalisation of hepatic metabolism, including the mobilisation of hepatic lipid deposits once THRT starts and weight loss begins. Removal of excessive fat from the liver is likely to improve the cholestasis which is typical of hepatic infiltration irrespective of the cause. This process may reasonably be expected to take some time to occur and therefore it is not surprising that the visit two ALKP concentration was not significantly different from the pre-treatment value.

Circulating AST concentrations were significantly decreased at visits three and four compared to visit one, and at visit four compared to visit two. However, the numerical changes were small and would be difficult to interpret in an individual case. There are multiple sources of circulating AST in dogs, including red blood cells and hepatic, cardiac and muscular tissue (Willard, 1989). Possible explanations for the decrease in concentration with THRT are therefore varied but include resolution of hepatic lipid deposits as discussed above, and/or resolution of subclinical myopathic disease (Braund *et al.,* 1981). The absence of any significant change in circulating CK concentration following the start of THRT suggests that muscle is less likely to be the source of the AST.

An increase in circulating CK values is traditionally considered to occur in canine hypothyroidism but as detailed in Chapter 5, there was no significant difference between CK values in hypothyroid compared with euthyroid dogs with various NTI in the present study. It is therefore not surprising that there was no statistically significant change in CK values in dogs after starting THRT. These findings support the conclusion that circulating CK measurement is of little diagnostic utility in either confirming or monitoring therapy for canine hypothyroidism.

The RBC count was significantly increased on every visit when compared with the median result from the previous visit. This confirms reversal of the physiological anaemia which is associated with hypothyroidism in humans and dogs (Green & Ng, 1986). Like the change in fructosamine, RBC count decreased between the final two visit periods, indicating that it may not have stabilised by the end of the study. Inspection of the data revealed that the rate of increase in RBC count was fairly linear throughout the study and no plateau was identified. This indicates that the haematological changes would probably have continued to improve although a longer follow up period would be necessary to confirm this. The fact that RBC count increased within two weeks of commencing THRT is useful and indicates that RBC measurement may be of some value in monitoring these cases throughout the stabilisation period. However, as discussed for other analytes, analysis of samples from a larger group of dogs, and correlation of these clinicopathological changes with clinical response will be needed to confirm statistically useful parameters.

Circulating Hb values were significantly increased at all visits compared with visit one, and at visits three and four compared with visit two. The changes and mechanisms responsible for these changes are as described for the RBC count. The changes which occurred in MCH were likewise considered to be simply a reflection of the alteration in haemoglobin concentration. The MCV value was significantly reduced at visit four compared to all previous visits. This was not considered to be of any biological significance, although the cause of the mild reduction was not apparent.

It is clear that a number of routine biochemical and haematological parameters change significantly following the start of THRT. There is also variation in the time taken for individual parameters to start improving, and the period over which these changes occur. The results of this study indicate that in the early stages of therapy, cholesterol, creatinine and triglyceride concentrations may be of most value in confirming a clinicopathological response to treatment. With more protracted therapy, ALKP, AST, albumin and globulin results appear to be good indicators that metabolism is normalising.

Both RBC count and fructosamine concentration are markers which show rapid and continued improvement throughout the course of treatment, and further study of these analytes in particular is warranted.

There was a significant decrease at visit two in circulating creatinine concentration in those cases which needed no increase in THRT at any point during the study compared with those cases in which an increase was required at some time. This indicates that whilst monitoring of certain parameters as detailed above allows confirmation of biochemical and haematological improvements, the estimation of creatinine in particular provides for a rapid and inexpensive method of predicting the adequacy of THRT within as little as two weeks after starting therapy. This finding has not been previously reported and because of the time and expense implications, may prove to be an invaluable aid in the routine monitoring of canine hypothyroidism. No evaluation was made of potential markers of THRT over-supplementation due to the small number of cases in this category.

With the exception of creatinine, none of the routine clinicopathological parameters were significantly different at visit two between adequately treated and under-treated cases. The similar biochemical and haematological response between these two groups, despite the variable clinical response and requirement for dosage adjustments, confirms the need for a therapeutic regime tailored to the specific clinical requirements and not clinicopathological features of individual dogs.

In conclusion, good clinical and clinicopathological control of hypothyroid dogs can be achieved in the vast majority of cases with once daily THRT. Using a starting dose of 0.02 mg/kg, approximately half of all cases will need a single dosage adjustment within the first 12 weeks and be adequately stabilised on a mean dose of approximately 0.028 mg/kg L-T_4. It is rare for more than one dose adjustment to be required in any individual. A mean six hour post pill peak total T_4 concentration of approximately 55 nmol/L provides good clinical control in most cases, and alongside cTSH estimation, can be measured within approximately two weeks of starting therapy, or presumably also after making a dosage adjustment. A peak total T_4 concentration of approximately 35 nmol/L is a reasonably reliable cut off which can be used as to indicate that an increase in dose is likely to be clinically required in an individual. This provides guidelines which may be adopted in future studies. There is a rapid improvement in certain clinical characteristics including weight reduction which occurs within approximately two weeks of commencing THRT. By approximately three months after starting THRT, a mean weight reduction of 10 % can be expected. Other clinical improvements are variable but typically lethargy and mental

demeanour are the first signs to indicate successful treatment, usually improving within two weeks of starting THRT. Other clinical improvements have been summarised. Routine clinicopathological monitoring is of value in confirming a general metabolic response to THRT but is of limited value in accurately monitoring cases or tailoring therapy. The exception is estimation of circulating creatinine which significantly reduces in dogs who are adequately treated compared with those who are likely to require subsequent dosage adjustments within two weeks of starting THRT.

CHAPTER 7

CIRCULATING THYROGLOBULIN AUTOANTIBODIES IN HYPOTHYROID AND EUTHYROID DOGS

7.1. LITERATURE REVIEW

Thyroid Pathology in Hypothyroidism

In humans, chronic autoimmune thyroiditis is the original paradigm for autoimmune diseases in general. This term encompasses a variety of different entities including goitrous (Hashimoto's), atrophic, juvenile, postpartum, silent and focal thyroiditis although the interrelationship of some of these remains unclear. In practice many of these terms are used synonymously with autoimmune thyroiditis. The disorder, which is most commonly recognised in women, encompasses a range of clinical and pathological changes from asymptomatic pathology detected post mortem, to extensive infiltrative inflammation and scarring with complete loss of normal thyroid tissue. The pathology typically consists of varying degrees of lymphocytic infiltration, fibrosis and loss of follicular epithelium. It is estimated that approximately half of all cases of naturally occurring canine primary hypothyroidism result from lymphocytic thyroiditis whereas the remainder result from idiopathic thyroidal atrophy (Gosselin, Capen, Martin & Krakowka, 1982).

Gosselin *et al.,* (1981a) evaluated the thyroid glands from 16 dogs with spontaneous hypothyroidism using light and electron microscopy. Dogs were confirmed as hypothyroid based on the presence of appropriate clinical signs and an inadequate response to TSH administration. In seven of the 16 (44 %) cases lymphocytic thyroiditis was confirmed which was characterised by diffuse infiltration of the thyroid gland by lymphocytes, plasma cells and macrophages. There was formation of lymphoid nodules and evidence of follicular destruction progressing to replacement of most of the gland with fibrous connective tissue. The basement membrane around the follicles contained electron dense deposits and the authors concluded that the pathology observed was immune-mediated in origin. In the remaining nine (56 %) dogs, idiopathic follicular atrophy was confirmed, characterised by loss of thyroid parenchyma and replacement with adipose connective tissue. Follicular cell degeneration with exfoliation into the colloid and interfollicular areas was present. Most of these thyroid glands were composed largely of adipose tissue interspersed with either small follicles or individual follicular cells. The remaining follicular tissue had dilated rough endoplasmic reticulum, large golgi apparatus

and intracytoplasmic microfollicles. The authors concluded that this pathology was a degenerative lesion of the follicular cells although the cause remained unclear. The condition was not associated with an inflammatory component.

Lucke *et al.*, (1983) evaluated the thyroid glands of seven dogs with clinical signs consistent with hypothyroidism by light and electron microscopy. No further details regarding the diagnostic criteria used to confirm hypothyroidism were provided. The pathology observed in six of these cases was similar although varied in severity. Focal to diffuse infiltration of the follicular epithelium, interfollicular tissue and colloid with lymphocytes, plasma cells and macrophages, with destruction and replacement of follicles was reported. In five of these six animals, there were relatively few normal follicles remaining. Most follicles that were left were small and lined by enlarged cuboidal cells containing vesicular nuclei and granular eosinophilic cytoplasm. In contrast to Gosselin *et al.*, (1981a), fibrosis was not reported to be a feature of the thyroid glands in any of the cases with inflammatory infiltrate. However, in the sixth case there was minimal inflammatory cell infiltrate but striking fibrosis. The authors suggested that this may have been the end stage of an inflammatory process although no further evidence for this conclusion was provided.

Chastain, Young & Kemppainen, (1989) confirmed hypothyroidism in a dog with T_3 autoantibodies based on the results of a TSH response test. Histopathological examination of the thyroid gland revealed replacement of approximately 50 % of the normal gland with adipose tissue. The remaining tissue contained collapsed thyroid follicles with low cuboidal epithelium. The follicular cell cytoplasm was pale and vacuolated, and scattered aggregates of lymphocytes and plasma cells were observed in the interstitium. The histological diagnosis was concurrent lymphocytic thyroiditis and thyroid atrophy. Strangely, the authors made no comment regarding the concurrent presence of both idiopathic atrophy and lymphocytic thyroiditis in this case.

Aetiology of Thyroiditis

Broadly speaking, autoimmune diseases occur when there is a failure of T-cell tolerance as a result of both genetic and non-genetic factors. The aetiopathogenesis of autoimmune thyroiditis in humans has been extensively studied and both genetic and non-genetic influences play a role. Factors involved in the development and progression of the disease may include infectious agents, dietary factors, toxins, hormones, stress and major histocompatability complex (MHC) and cytokine regulatory genes (Weetman, 1996). Like

the human disease, lymphocytic thyroiditis in the dog is also considered to be immune mediated (Beale, Halliwell & Chen, 1990). As in humans, a strong genetic predisposition has been proposed and is supported by the demonstration of lymphocytic thyroiditis in particular breeds and families of dogs including borzois, great Danes, and beagles (Haines *et al.,* 1984b; Conaway *et al.,* 1985; Benjamin *et al.,* 1996). The largest published study was conducted by Benjamin *et al.,* (1996) who reported lymphocytic thyroiditis in 26.3 % of 276 inter-related control beagles and demonstrated a high degree of heretability for hypothyroidism. Nevertheless the underlying defect, or more likely defects, remains unclear.

Thyroiditis and Circulating Autoantibodies

The spontaneous development of circulating antibodies to antigenic thyroid tissues as a result of AITD was first documented by Roitt, Doniach, Campbell & Hudson, (1956) who reported the presence of TgAb in human patients with Hashimoto's thyroiditis. Since then numerous antibodies directed against various thyroidal components have been identified in association with human AITD, including thyroid peroxidase (TPO) autoantibodies (TPOAb), TSH receptor antibodies (TRAb), T_4Ab, and T_3Ab (McKenzie & Zakarija, 1996). Of most clinical impact was the identification of TPOAb by Trotter, Belyavin & Waddams, (1957) which have subsequently been shown to be more prevalent and present in higher titres than TgAb. TPOAb estimation is currently considered to be the most cost effective means of confirming the presence of AITD in humans (McKenzie & Zakarija, 1996).

The diagnostic utility of TPOAb estimation in the dog has been investigated previously. Haines, Lording & Penhale, (1984a) documented TPOAb in 10 of 34 (29 %) clinically hypothyroid dogs using an ELISA method. However, although the authors concluded that the technique may be useful as a screening aid, antibody titres were low and the method was not considered to be sufficiently sensitive or reliable to justify routine diagnostic use. More recently, Thacker, Davis, Refsal & Bull, (1995) examined sera from 50 dogs with autoantibodies to thyroglobulin, T_4 or T_3. All samples were negative for TPOAb. In addition no cross reactivity with human and porcine TPO was identified using this method, which was developed using purified canine TPO. Given the widespread clinical application of TPOAb estimation in human AITD, the authors concluded that the pathogenesis of lymphocytic thyroiditis was different between humans and dogs.

Experimental studies in dogs have confirmed that the intrathyroidal injection of TgAb is swiftly followed by the development of lesions within the gland similar to naturally occurring lymphocytic thyroiditis (Gosselin, Capen, Martin & Krakowka, 1981b). There is little doubt that TgAb is involved in the pathogenesis of canine thyroiditis but its exact role in the promotion of the disease remains unclear. The measurement of circulating canine TgAb is discussed in more detail below.

Currently the only other autoantibodies recognised in association with canine thyroiditis are T_4Ab and T_3Ab. Young, Sartin & Kemppainen, (1985) reported an abnormal "T_3 binding factor" which interfered with laboratory measurement of total T_3 in 18 (< 0.3 %) of over 6000 samples submitted to a commercial veterinary laboratory for evaluation of thyroid function. The presence of a T_3-binding factor was suspected in those samples with markedly increased apparent total T_3 concentrations. Binding of T_3 by the unknown factor was diminished by the addition of exogenous T_3 and could be precipitated by the addition of goat anti-dog IgG. Scatchard analysis demonstrated that the characteristics of the binding factor were similar to that of human T_3 autoantibodies and the authors concluded that canine T_3 autoantibodies were responsible for the anomalous increase in apparent total T_3 concentrations.

Subsequently, Thacker, Refsal & Bull, (1992) developed assays for TgAb, T_4Ab and T_3Ab. The TgAb assay developed was an ELISA method utilising purified canine thyroglobulin coated to the plate wells, and goat anti-dog IgG conjugated with alkaline phosphatase. Negative reference sera were obtained from 13 healthy dogs with reference range circulating thyroid hormone concentrations and normal thyroid gland histological findings. Two further dogs were repeatedly immunised with purified canine thyroglobulin in adjuvant and serum samples from these dogs were designated as the positive controls. An agarose gel electrophoresis assay was used to detect T_3Ab and T_4Ab. Positive control samples were obtained from one dog with increased total T_4 and decreased total T_3 concentrations on RIA, consistent with autoantibodies to T_3 and T_4. The authors examined serum from 119 dogs with clinical signs consistent with hypothyroidism and reported the presence of TgAb, T_3Ab and T_4Ab in 50 (42 %), 39 (33 %) and 31 (26 %) samples respectively. Autoantibodies to at least one of the three antigens were present in 58 (49 %) of the cases. The prevalence of T_3Ab and T_4Ab in the samples in this study is considerably greater than in subsequent studies but the reason for this is unclear.

Gaschen *et al.*, (1993) progressed this work and revealed that as well as being associated with apparent elevations in serum total T_3 concentration, the presence of T_3Ab

was significantly associated with the presence of TgAb. Circulating T_3Ab were measured by incubation of sample with radiolabelled T_3 followed by the addition of dextran-coated charcoal. After centrifugation, the radioactivity remaining in the supernatant was determined and compared to standards prepared from mouse monoclonal T_3Ab (Chrystal chemicals). Circulating T_3Ab was measured in serum samples from 45 dogs with increased TgAb concentrations and two distinct groups were identified. Low T_3Ab concentration occurred in 28 of the samples, whereas the remaining 17 had markedly increased T_3Ab concentrations. There was a direct linear correlation between apparent total T_3 and T_3Ab concentration. Furthermore, the addition of T_3 to TgAb containing sera, caused a significant reduction in the measurable TgAb concentration. The authors concluded that T_3Ab were most likely generated against specific epitopes present within the thyroglobulin molecule and that like TgAb, T_3Ab are a valid marker for canine thyroiditis.

Rajatanavin, Fang, Pino, Laurberg, Braverman, Smith & Bullock, (1989) measured circulating TgAb in 19 hypothyroid and 20 euthyroid dogs with a sensitive radioimmunoprecipitation assay, and T_3Ab and T_4Ab by polyacrylamide gel electrophoresis. Dogs were categorised based on clinical signs and total iodothyronine concentrations. A marked association between the presence of TgAb with both T_3Ab and T_4Ab in canine sera was reported.

Refsal & Nachreiner, (1997a) reported the prevalence of T_3Ab only, T_4Ab only and both autoantibodies in 4.4, 0.2 and 0.7 %, respectively, from approximately 240,000 samples submitted for thyroid hormone analysis to a veterinary laboratory. The presence of autoantibodies was highly associated with samples with increased cTSH or decreased thyroid hormone concentrations. Autoantibodies were most common in dogs between two and four years of age and in those > 10kg body weight. Certain breeds were more commonly affected, particularly the English setter, old English sheepdog, boxer, Brittany spaniel and dalmatian.

It is now apparent that both T_4 and T_3 autoantibodies are rarely present in samples without concurrent TgAb and that T_3Ab is more frequently identified than is T_4Ab. The prevalence of these iodothyronine autoantibodies in only a subset of dogs with TgAb has caused most of the recent research to be directed at the identification of TgAb. However, the practical importance of T_4Ab and T_3Ab lies in their potential to interfere with most widely used immunoassays for total T_4 and total T_3. If the autoantibody titre is sufficiently high, it can bind a significant proportion of the radiolabelled hormone used in the RIA procedure. If the labelled hormone-autoantibody complex appears in the free fraction of the

RIA, as occurs with most solid phase techniques, the apparent endogenous hormone concentration will be spuriously increased. This occurs with most of the widely used thyroid hormone assays (Chastain *et al.,* 1989; Thacker *et al.,* 1992; Kemppainen, Young, Behrend, Clark & Smiley, 1996). However, if the labelled hormone-autoantibody complex appears in the bound fraction of the RIA, such as with charcoal methods or double antibody assays in which the second antibody precipitates the complex, the value will be spuriously decreased.

Canine Thyroglobulin Autoantibody Estimation

Since it has become clear that TPOAb estimation is of minimal utility in the investigation of canine thyroid disease veterinary interest has centred principally on the estimation of circulating TgAb, first identified in dogs by Beierwaltes & Nishiyama, (1968). Subsequently, various methods, mostly modifications of human assays have been used for TgAb identification. However, the difference in methodologies has been responsible for markedly differing results between reports.

Gosselin *et al.,* (1980) reported TgAb in 12 of 25 (48 %) hypothyroid dogs using a chromic chloride passive haemagglutination (CCH) method but only 6 (24 %) of these 25 animals were positive by tanned cell haemagglutination (TCH). Using the CCH method five of 40 (12.5 %) dogs with various NTI were TgAb positive but all were negative using TCH. Haines *et al.,* (1984a) reported TgAb in 20 (59 %) and 18 (53 %) of 34 hypothyroid dogs using ELISA and CCH methods respectively but there was marked differences in the positive results between methodologies with 12 of the cases being positive by one method but not the other. Using the same ELISA method, Haines *et al.,* (1984b) reported TgAb in 28 of 65 (43 %) dogs with non-thyroidal endocrine diseases, 140 of 1057 (13 %) hospitalised patients with non-endocrine diseases and 30 of 64 (47 %) healthy dogs closely related to TgAb positive hospitalised patients. Vollset & Larsen, (1987) reported measurable TgAb in only 11 of 45 (24 %) and 15 of 44 (34 %) suspect hypothyroid dogs using glutaraldehyde (GCH) and CCH methods respectively, and only three of 42 (7 %) dogs with various NTI including endocrine diseases by both methods.

Recently a commercial TgAb ELISA kit has become available (Canine thyroglobulin autoantibody immunoassay kit, Oxford Laboratories). Following clinical validation results first presented by Graham, Nachreiner & Refsal, (1997) a more comprehensive evaluation of this assay was reported by Nachreiner *et al.,* (1998). Serum samples containing T_3Ab and T_4Ab, and therefore with a high potential for the presence of

TgAb, were evaluated for TgAb and compared to those without iodothyronine autoantibodies to determine the optimal diagnostic cut-off of the assay for confirming the presence of TgAb. An o.d. value equal to or greater than 200 % of the negative control sample was associated with an overall test efficiency of 0.984 and was subsequently employed. There was good correlation of the TgAb result with thyroid biopsy findings in eight dogs with, and 10 dogs without histological evidence of thyroiditis. Some of these cases had mild focal thyroiditis whereas others had more severe thyroid pathology. The cases with mild lymphocytic infiltrate typically had low cuboidal follicular epithelial cells, consistent with normal thyroid function. However, those cases with more severe and diffuse infiltrates had tall columnar epithelial cells consistent with reduced thyroid function and increased cTSH concentrations. The implication of this finding is that increased circulating TgAb can potentially be used as a marker of thyroid disease, prior to the development of functional thyroid failure. In the same study, the prevalence of TgAb in random source dogs was evaluated by TgAb estimation in 91 apparently healthy dogs. Three cases (3.3 %) were positive for TgAb. Additional investigation of two of these three cases demonstrated decreased circulating free T_4 and increased cTSH concentrations consistent with primary hypothyroidism. In one of these dogs, thyroid gland biopsy was confirmatory of lymphocytic thyroiditis. The third dog was not evaluated further. The prevalence of TgAb in 131 dogs with hypothyroidism was also evaluated in the same study. Cases were confirmed as hypothyroid based on demonstration of increased circulating cTSH and decreased iodothyronine concentrations. However, only cases in which autoantibodies to both T_3 and T_4 were absent were included in the study. In total, 50 of the 131 (38.2 %) hypothyroid cases were TgAb positive. When hypothyroid dogs including those with T_3 and T_4 autoantibodies were also included, TgAb was present in 87 of 171 (51%) cases. The mean (± s.d.) age of the TgAb positive and -negative hypothyroid dogs was 6.1 ± 2.5 years and 7.1 ± 3.0 years, respectively. The authors concluded that this may be consistent with a progressive change from TgAb positivity to TgAb negativity as thyroiditis progresses. The prevalence of TgAb was also evaluated in 146 dogs with various NTI and was positive in eight (5 %). The non-thyroidal disease categories included calcium disorders, hyperadrenocorticism, diabetes mellitus, hypoadrenocorticism and obesity. Twenty three of 24 (96 %) samples with T_3Ab were positive for TgAb, whereas all of 37 samples with otherwise normal indices of thyroid function were negative for the autoantibody.

Using the same assay, Graham, Refsal & Nachreiner, (1998) studied nine laboratory beagles, five of which exhibited various states of thyroid pathology. Three distinct categories were identified based on TgAb status, cTSH and total T_4 concentrations and severity of the thyroid lesions. Subclinical thyroiditis was identified in two cases, defined as the presence of mild focal thyroiditis and increased TgAb but otherwise unremarkable results. Subclinical hypothyroidism was identified in two cases, defined as the presence of severe diffuse thyroiditis, increased TgAb and circulating cTSH concentration but normal total T_4 concentration. Clinical hypothyroidism was present in one dog, defined as severe lymphocytic thyroiditis, increased TgAb and circulating cTSH concentration and decreased total T_4 concentration. TSH response tests were performed in each of the dogs and were consistent with the extent of the thyroid pathology. Specifically, the total T_4 response to TSH was significantly decreased compared with the healthy cases but the authors reported that the results were not low enough to be considered hypothyroid by conventional criteria. However, these data were not provided. Prolonged observation of these beagles has also revealed that two have progressed between stages presumably supporting the dynamic nature of lymphocytic thyroiditis in the dog. However, the relevant time period during which this transition occurred was not specified.

Using the same assay, Nachreiner, Bowman, Graham, Refsal & Bolliger, (1999) evaluated TgAb status in 51201 samples submitted for investigation of possible hypothyroidism. The overall prevalence of TgAb positive results was 7.9 %. This prevalence was highest in dogs from two to six years of age and declined in dogs older than six years. There was no association of TgAb result with gender. The hypothyroid dogs (categorised based on subnormal circulating total and free iodothyronine and increased cTSH concentrations) which were TgAb positive were significantly younger (median age approximately five years) than those hypothyroid dogs which were TgAb negative (median age approximately eight years). The authors concluded that hypothyroidism resulting from idiopathic atrophy may therefore be a sequel to lymphocytic thyroiditis. The study identified a strong breed predisposition for the presence of circulating TgAb, with (prevalence of TgAb positive results within brackets) English setter (26 %), dalmation (17 %), basenji (16 %), Rhodesian ridgeback (16 %), old English sheepdog (15 %), boxer (14 %), Maltese terrier (14 %), Chesapeake Bay retriever (13 %), beagle (13 %), cocker spaniel (13 %) and Shetland sheepdog (13 %) being most significantly over-represented when compared to the prevalence of TgAb in mixed breed dogs. The authors concluded that the relationship between TgAb prevalence and breed, supports the hypothesis that

there is a strong genetic component to the development of thyroiditis and consequently hypothyroidism.

Siliart & Stambouli, (1997) previously assessed the same ELISA assay but with very different conclusions. Whilst the authors acknowledged the diagnostic errors associated with the TRH response test for confirming hypothyroidism, they used an unusual variation of it, based on intramuscular administration of TRH, to classify 665 dogs as hypothyroid. Circulating TgAb was determined in a group of 40 of these cases and four (10 %) had a positive TgAb result. The misclassification of euthyroid dogs as hypothyroid was likely responsible for the conclusions in the study. Misclassification would also explain the finding of circulating cTSH concentrations greater than 0.6 ng/ml in only 20 % of the "hypothyroid" dogs in that report which is at marked variance with all other contemporary reports as outlined in Chapter 4 (Williams *et al.,* 1996; Jensen *et al.,* 1996; Peterson *et al.,* 1997; Ramsey *et al.,* 1997; Scott-Moncrieff *et al.,* 1998).

Iverson *et al.,* (1998) developed and validated a separate TgAb ELISA assay to that reported by Nachreiner *et al.,* (1998). Canine thyroglobulin was harvested and purified from the thyroid glands of three healthy dogs for use in the assay. Three calibrator samples were included in each assay run. These included a positive sample from a dog with hypothyroidism and histologically confirmed lymphocytic thyroiditis, and two negative samples from two healthy dogs with no detectable clinical, haematological or biochemical abnormalities. In each assay, a K-value (equal to the o.d. of the positive calibrator minus the mean values from the negative calibrators) was calculated and used to calibrate the assay. All test samples were compared to the K-value and assigned an Ab value which was the percentage of the K value. The assay LoD was defined as the NSB plus twice the s.d. obtained from analysis of samples in duplicate with an Ab score less than 7.5 % on 13 occasions. The LoD was 5.5 % corresponding to an o.d. of 0.009. The intra-assay c.v.'s obtained with low, moderate and positive Ab score samples over 15 runs were 3.2, 4.9 and 2.0 %, respectively. The inter-assay c.v.'s obtained with low, moderate and high Ab score samples over 10 to 15 runs were 4.6, 7.3 and 9.9 %, respectively. The optimal cut-off value calculated by DPR analysis for the confirmation of thyroiditis was 57.4 %. A median Ab score in 132 healthy dogs was 10 % which was close to the LoD of the assay. The median Ab score in dogs with primary hypothyroidism and confirmed lymphocytic thyroiditis (n=11), skin diseases (n=35) and other NTI (n=63) was 340, 12 and 8 %, respectively. The diagnostic sensitivity of the assay for lymphocytic thyroiditis was 0.91 and the diagnostic specificity was 0.97. The prevalence of TgAb in healthy dogs and dogs with NTI was 5 and

6 %, respectively. The assay developed by Iverson *et al.*, (1998) has not yet become commercially available.

7.2. INTRODUCTION

In conjunction with appropriate clinical signs and clinicopathological abnormalities, evidence of thyroiditis as demonstrated by measurable circulating antibodies to thyroglobulin (TgAb) has been considered useful in supporting a diagnosis of hypothyroidism (Beale, 1991). However many TgAb measurement techniques previously available were cumbersome to use and in particular were associated with positive results in euthyroid dogs with NTI (Gosselin *et al.,* 1980; Haines *et al.,* 1984a; 1984b; Vollset & Larsen, 1987; Beale *et al.,* 1990; Beale, 1991). A commercial ELISA kit has recently become available for canine serum TgAb measurement (Canine thyroglobulin autoantibody immunoassay kit, Oxford Laboratories). This assay was recently evaluated by Nachreiner *et al.,* (1998) in order to determine a diagnostic threshold for the confirmation of thyroiditis. The purposes of this study were to more fully evaluate the potential value of this assay in the investigation of canine hypothyroidism by comparing results from clinically healthy dogs and those with confirmed hypothyroidism or NTI.

7.3. MATERIALS AND METHODS

Case Material

A series of 119 dogs referred for investigation of clinical or clinicopathological abnormalities consistent with hypothyroidism outlined in Chapter 2, and 70 apparently healthy dogs, were used for the study. Pedigree was classified as described in Chapter 2. The dogs were divided into either hypothyroid, euthyroid or healthy groups. The hypothyroid and euthyroid dogs were classified as described in Chapter 2. The hypothyroid group (mean age 7.4; range 3-12 years) contained 24 female (12 neutered) and 18 male (2 neutered) and the euthyroid group (mean age 7.4; range 1-14 years) contained 36 female (20 neutered) and 41 male (8 neutered) dogs. The healthy group consisted of 70 clinically healthy dogs. This group was divided into two subsets, group A consisting of 19 dogs of a variety of breeds (mean age 4.9, range 0.5-12 years) and group B consisting of 51 bearded collies (mean age 3.9, range 0.5-11 years) being sampled at the request of their owners as part of a breeding programme. Group A contained 10 female (6 neutered) and nine male (6 neutered) dogs and group B contained 32 female (5 neutered) and 19 male (2 neutered) dogs.

Hormone and Thyroglobulin Autoantibody Estimation

Blood samples were collected and stored for estimation of TgAb, cTSH, total T_4 and free T_4 as described in Chapter 2.

Statistical Analysis

The effect of thyroid status, sex, neutering and pedigree status on TgAb results was determined by chi-squared analysis. Comparison of dog ages and thyroid hormone concentrations between groups was performed using a two-sample t-test.

7.4. RESULTS

Hormone concentrations from each group are summarised in Table 19. The distribution of the major diseases in the euthyroid group is summarised in Table 20.

	*Healthy	Hypothyroid	NTI
Basal total T_4 (nmol/L)	24.3 ± 6.5	7.8 ± 4.5	22.2 ± 11.0
Post-TSH total T_4 (nmol/L)	ND	9.5 ± 6.8	62.3 ± 33.2
cTSH (ng/ml)	0.29 ± 0.22	2.2 ± 2.5	0.41 ± 0.46
free T_4 (pmol/L)	19.6 ± 5.5	5.4 ± 3.4	16.5 ± 9.25

ND Analysis not done

Table 19. Serum hormone results (mean ± s.d.) in healthy dogs and those with confirmed hypothyroidism or non-thyroidal illness (NTI).

*Results exclude two dogs subsequently confirmed as hypothyroid.

Disease Type	Number of Dogs
Dermatological	20 (26%)
Obesity	16 (21%)
Non-thyroidal endocrine diseases	10 (13%)
Neurological	8 (10%)
Neoplasia	6 (8%)
Cardiovascular	6 (8%)
Musculoskeletal	2 (3%)
Gastrointestinal	3 (4%)
Ophthalmological	2 (3%)
Other*	4 (5%)

Table 20. Principal disease categories affecting dogs with non-thyroidal illness (NTI). *The "other" category includes one case each of idiopathic tracheitis, vaginitis, nasopharyngeal foreign body and iatrogenic hyperadrenocorticism.

There was no significant difference in TgAb-positive, -equivocal or -negative results between groups A and B and therefore these were combined for analyses. Results of TgAb measurement in each of the three groups are summarised in Table 21 and displayed in Figures 23, 24 and 25.

Category	Number of Dogs	TgAb Result		
		Negative	Equivocal	Positive
Hypothyroid	42	19 (45%)	8 (19%)	15 (36%)
NTI	77	72 (94%)	5 (6%)	0 (0%)
Healthy	70	55 (79%)	11 (16%)	4 (6%)

Table 21. Distribution of circulating thyroglobulin autoantibody (TgAb) results in hypothyroid dogs, euthyroid dogs with non-thyroidal illness (NTI) and clinically healthy dogs.

There were significantly more TgAb-positive results in the hypothyroid group compared to the euthyroid and the healthy groups ($p<0.001$ in each case) and in the healthy group compared to the euthyroid group ($p=0.02$). There were significantly ($p=0.01$) more TgAb-equivocal results in the hypothyroid group compared to both the euthyroid group and to the euthyroid and healthy groups combined but there was no significant difference in the distribution of results between the euthyroid and the healthy group.

Four dogs in the healthy group were positive for TgAb. Three of these four dogs were entire female bearded collies and the remaining one was an entire male rottweiler. Basal thyroid hormone analyses from these dogs are presented in Table 22 and supported euthyroidism in two and hypothyroidism in two as evidenced by subnormal total T_4 and free T_4 and markedly elevated cTSH concentrations. One of the biochemically hypothyroid dogs had mild dermatological changes (slight scurfing) which resolved after the institution of THRT. The owners of the remaining hypothyroid dogs elected not to treat the case since no clinical abnormalities were apparent. No clinical abnormalities have subsequently developed in this latter case during a follow up period of 15 months.

Animal	Basal total T_4 (nmol/L)	free T_4 (pmol/L)	cTSH (ng/ml)
BC 09	10.9	4.11	11.3
BC 14	30.4	16.96	0.46
BC 15	14.4	4.74	9.31
RW 1	19.0	13.28	0.19

Table 22. Concentrations of serum basal total and free thyroxine (T_4), and thyrotropin (cTSH) in apparently healthy but thyroglobulin autoantibody (TgAb) positive dogs.

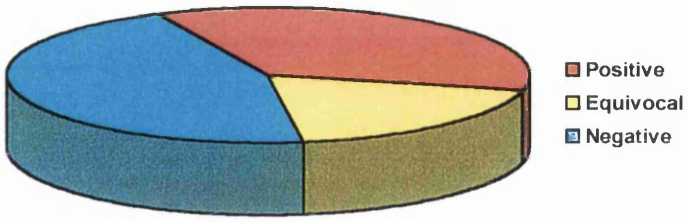


Figure 23. Distribution of circulating thyroglobulin autoantibody (TgAb) results from 42 dogs with confirmed hypothyroidism.

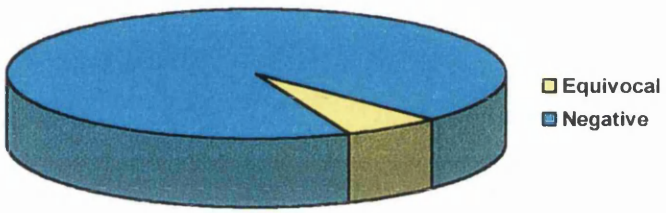


Figure 24. Distribution of circulating thyroglobulin autoantibody (TgAb) results from 77 dogs investigate for hypothyroidism but in which an alternative diagnosis was confirmed.

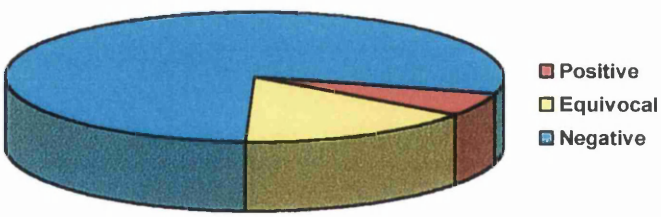


Figure 25. Distribution of circulating thyroglobulin autoantibody (TgAb) results from 70 clinically healthy dogs.

There was no significant difference in age, sex or neutering status between TgAb-positive and -negative animals within each group although the TgAb-positive hypothyroid dogs (mean age 6.6, range 3.0-10.6 years) tended (p=0.09) to be younger than the TgAb-negative hypothyroid individuals (mean age 8.1, range 4.0-12.0 years).

The most commonly represented breeds and breed-types and the distribution of TgAb results within each category are summarised in Table 23. There was no statistical difference in the distribution of TgAb positive, equivocal or negative results when grouped according to breed-type.

Breed	Number	TgAb Result		
		Positive	Equivocal	Negative
Collies	63	4	10	49
Retrievers	32	6	4	22
Terriers	20	1	3	16
Crossbreeds	13	1	2	10
Spaniels	11	0	1	10
Boxer	5	1	0	4
Doberman	5	0	0	5
Setters	5	0	0	5

Table 23. Distribution of thyroglobulin autoantibody (TgAb) results categorised by breed-type from hypothyroid dogs, dogs with non-thyroidal illness (NTI), and clinically healthy dogs. Only breed-types with at least five cases are presented.

The distribution of pedigree and non-pedigree status in the hypothyroid, euthyroid and healthy groups is detailed in Tables 24, 25 and 26, respectively. There was no significant difference in the prevalence of TgAb-positive results between pedigree and non-pedigree dogs either within each group or between groups, respectively.

	Pedigree	Non-Pedigree
TgAb Positive	14	1
TgAb Equivocal	7	1
TgAb Negative	17	2
Total	**38**	**4**

Table 24. Thyroglobulin autoantibody (TgAb) results obtained from 42 hypothyroid dogs categorised by pedigree status.

	Pedigree	Non-Pedigree
TgAb Positive	0	0
TgAb Equivocal	5	0
TgAb Negative	67	5
Total	**72**	**5**

Table 25. Thyroglobulin autoantibody (TgAb) results obtained from 77 dogs with confirmed non-thyroidal illness (NTI) categorised by pedigree status.

	Pedigree	Non-Pedigree
TgAb Positive	4	0
TgAb Equivocal	10	1
TgAb Negative	46	9
Total	**60**	**10**

Table 26. Thyroglobulin autoantibody (TgAb) results obtained from 70 apparently healthy dogs categorised by pedigree status.

There was no significant difference in basal total T_4, cTSH or free T_4 concentrations between TgAb-positive, -negative or -equivocal dogs within the hypothyroid or euthyroid groups, respectively. There was no significant difference in hormone results between TgAb-negative and –equivocal dogs within the healthy group. Statistical analyses were not performed in TgAb-positive animals within this group because of subsequent confirmation of hypothyroidism in two of the four animals.

Two of the hypothyroid TgAb positive dogs were subsequently euthanatised for reasons unrelated to their thyroid disease. In each case, post-mortem examination, including thyroid gland histopathological examination was performed. One case was an 11 year old entire male boxer euthanatised as a result of a pancreatic carcinoma. The thyroid glands from this dog were grossly small and pale. Histopathological examination of the thyroids revealed diffuse multifocal lymphocytic thyroiditis consistent with the TgAb result. The second dog was an eight year old entire male border terrier euthanatised as a result of encephalitis of unknown aetiology. The thyroid glands from this dog were grossly small and pale. Histopathological examination of the thyroids revealed diffuse thyroidal atrophy and replacement of normal tissue with adipose tissue. No inflammatory infiltrate was present. A diagnosis of idiopathic atrophy was made in this case.

All three of the euthyroid, and one of the four hypothyroid dogs with diabetes mellitus in whom TgAb was measured were negative for the autoantibody. The remaining three hypothyroid cases with concurrent diabetes mellitus were TgAb positive.

In seven hypothyroid dogs with reference range cTSH concentrations, TgAb was positive in three (43 %), equivocal in one (14 %) and negative in three (43 %) of them. In nine euthyroid dogs with elevated cTSH concentrations TgAb was equivocal in one (11 %), and negative in eight (89 %) of them.

7.5. DISCUSSION

The demonstration of TgAb in over a third of hypothyroid dogs is broadly similar to previous reports (Gosselin *et al.,* 1980; Haines *et al.,* 1984a; Vollset & Larsen, 1987). However in those studies whilst the overall percentage was similar to the results reported here, there was considerable method-dependent variation between individual cases which reduced the diagnostic utility of those assays. Using the same assay as the present study, Nachreiner *et al.,* (1998) reported TgAb in just over half of 171 hypothyroid dogs. However some of those animals were specifically selected for the presence of thyroid

hormone autoantibodies which would bias the results in favour of TgAb positivity. The true prevalence of TgAb in hypothyroid dogs is therefore likely to be nearer that reported in the present study.

None of the dogs with NTI, including those with non-thyroidal endocrine diseases, were positive for TgAb although five (6 %) were equivocal. These equivocal cases were spread between cardiovascular (n=2), dermatological (n=1), endocrinological (n=1), and neurological (n=1) diseases. In comparison with the non-thyroidal diseases evaluated by Nachreiner *et al.,* (1998) no euthyroid dogs in the present study had recognised calcium disorders although cases with hyperadrenocorticism (n=4), diabetes mellitus (n=4), obesity (n=9) and hypoadrenocorticism (n=2) were present and negative for TgAb. The high prevalence of TgAb positive results associated with non-thyroidal diseases previously reported does not appear to be a significant problem with the current assay (Haines *et al.,* 1984a; 1984b). This supports the conclusions of Nachreiner *et al.,* (1998), confirming the much improved specificity of this assay for thyroid disease compared with previous methods.

TgAb-positive and -equivocal results occurred in four (6%) and 11 (16%) of 70 apparently healthy dogs, respectively. Two of these TgAb-positive dogs had additional biochemical evidence of hypothyroidism as did two of three apparently healthy TgAb-positive dogs reported by Nachreiner *et al.,* (1998). This suggests that the presence of circulating TgAb in apparently healthy animals could be an early indicator of thyroid disease as it is in humans (Gordin & Lamberg, 1975). Of particular interest was the extreme elevations in cTSH concentrations found in two of the four cases in the present study which approximated 6-7 times that commonly encountered in spontaneous primary hypothyroidism. It is therefore plausible that very elevated circulating cTSH concentrations may occur in the early, possibly pre-clinical stages of thyroiditis, as the functional capacity of the thyroid gland begins to fall. However, because biochemical euthyroidism was demonstrated in the remaining two dogs, a TgAb-positive result should not be presumed to coexist with thyroid dysfunction. Certainly, caution is advised in over-interpretation of such results and additional clinical and clinicopathological monitoring for hypothyroidism is required to allow a confident diagnosis. In a previous study, two of eleven TgAb-positive euthyroid dogs followed for 18 months developed biochemical but not clinical evidence of hypothyroidism (Haines *et al.,* 1984b) although other similar cases showed no such progression during this time. The possible subsequent development of hypothyroidism in

TgAb-positive healthy dogs could not be supported or refuted in the present study because of limited follow-up information. Further study is undoubtedly required in this area.

The importance of TgAb-equivocal results was not addressed by either Siliart & Stambouli, (1997) or Nachreiner *et al.*, (1998). The TgAb assay manufacturers recommend re-testing equivocal patients at a later date, although there is no data available to suggest whether such an individual is likely to become TgAb-positive or –negative with time. In the present study, thyroid hormone concentrations were not significantly different in TgAb-equivocal, -negative or –positive cases within each group, which is not supportive of a progressive change in TgAb status from negative through equivocal to positive as the transition from euthyroidism to hypothyroidism occurs. However, such results may be influenced by the relatively small number of cases within each category. TgAb-equivocal results were significantly more prevalent amongst hypothyroid than euthyroid dogs, suggesting that it may be prudent to undertake further investigation of thyroid function in TgAb-equivocal dogs. Lengthier longitudinal case studies will help in clarifying the association of TgAb with the stage of thyroid disease.

The TgAb-positive hypothyroid dogs tended to be younger than the TgAb-negative hypothyroid supporting the results of Nachreiner *et al.*, (1998). This may be related to the earlier onset of hypothyroidism in individuals at risk from lymphocytic thyroiditis (Feldman & Nelson, 1996). Alternatively the results may be consistent with a progression from TgAb positivity to -negativity with increasing age and disease progression. Nachreiner *et al.*, (1998) identified a similar trend and suggested it was indicative of a progression of cases from lymphocytic thyroiditis in the earlier stages of disease, to idiopathic atrophy in the latter (Nachreiner *et al.*, 1999). Whilst this is certainly a possibility, the TgAb results themselves are not confirmatory of such a progression and appropriate longitudinal studies may help to clarify this issue.

There was no evidence of sex or neutering status influencing TgAb which agrees with the findings of previous studies (Haines *et al.*, 1984b; Vollset & Larsen, 1987; Beale *et al.*, 1990). This also supports the findings in Chapter 5 in which no influence of sex or neutering status on the presence of hypothyroidism was identified.

Previous studies have documented thyroiditis within particular breeds and families of dogs (Haines *et al.*, 1984b; Conaway *et al.*, 1985). However whilst individual families were not evaluated, no such breed effect was identified in the present study. Although several breeds were present in sufficient number to allow statistical analysis, the analysis was limited by the small number of cases of each breed within the hypothyroid category

specifically. This decreased the likelihood of identifying a genuine breed-predisposition since the hypothyroid dogs were represented by a large number of different breeds. Of the breed types studied, retrievers had the greatest percentage of TgAb positive results (19 %) as a proportion of the total number evaluated. Although this was markedly larger than the next highest value (6 %) which occurred in collies, the finding was not statistically significant. Evaluation of a larger number of affected dogs is likely to clarify the issue regarding the influence of breed on the development of thyroiditis and hypothyroidism.

There was no evidence that pedigree status had a significant effect on the prevalence of abnormal TgAb results. As was the case for statistical analysis of breed type, evaluation of the effect of pedigree was limited by the small number of dogs in the non-pedigree categories. Whilst the distribution of pedigree and non-pedigree cases could not have been predicted before commencement of this study, it is likely that considerably larger numbers of cases will be required, including the analysis of specific families of dogs to further evaluate this subject.

Of particular interest is the confirmation of idiopathic atrophy in a TgAb positive dog. This finding is in contrast to the results of Nachreiner *et al.*, (1998) in which TgAb was negative in 10 dogs without histopathological evidence of lymphocytic thyroiditis. One possible explanation for this finding is that lymphocytic thyroiditis and TgAb production precede the development of idiopathic atrophy in a number of hypothyroid dogs. This would be consistent with the finding of TgAb positive results in younger hypothyroid dogs than those with TgAb negative results. However, this would not necessarily explain the discordancy between the results of the present study and that of Nachreiner *et al.*, (1998) and the reason for this finding remains unclear. The possible explanations for this finding in the present study are undoubtedly worthy of further study.

TgAb was measured from four of the five hypothyroid dogs with concurrent diabetes mellitus discussed in Chapter 5. Of these four cases, TgAb was positive in three and negative in one of them. In contrast, three dogs in the euthyroid group with diabetes mellitus were all negative for TgAb. Whilst the numbers of cases are inadequate to allow meaningful statistical analysis, this findings may be consistent with the presence of an autoimmune polyglandular syndrome as the cause of the concurrent hypothyroidism and diabetes mellitus in the three TgAb positive dogs (Dixon, 1998).

Serum cTSH is now routinely measured alongside total T_4 or free T_4 to establish a diagnosis of hypothyroidism and these data have been presented in Chapter 4. However a subset of hypothyroid dogs have reference range cTSH concentrations and euthyroid dogs

can have cTSH results within the hypothyroid range (Peterson *et al.,* 1997, Scott-Moncrieff *et al.,* 1998). TgAb measurement correctly identified thyroid abnormalities in 43% of hypothyroid dogs with reference range cTSH concentrations. This confirms the value of TgAb measurement in these cases because whilst a negative TgAb result cannot exclude hypothyroidism a positive result strongly supports it. In addition, the presence of TgAb in a similar proportion of hypothyroid dogs with the expected rise in serum cTSH concentration to those with unexpectedly "normal" cTSH results is strong evidence that central hypothyroidism is not a common cause of such discordant results.

CHAPTER 8

THE EFFECT OF NON THYROIDAL ILLNESS AND DRUG THERAPY ON TESTS OF THYROID FUNCTION

8.1 LITERATURE REVIEW

Introduction

The confusion surrounding the interpretation of thyroid function tests in veterinary medicine has possibly been the most important reason why the diagnosis of canine hypothyroidism remains problematic. Much of the confusion results from the influence that NTI and a variety of commonly used drug therapies have on these tests (Panciera, 1990b; Feldman & Nelson, 1996). Whilst there is normally a degree of overlap in the results obtained from healthy and hypothyroid dogs, this overlap is considerably greater between euthyroid dogs with NTI or those dogs receiving certain therapies, and those with hypothyroidism. Additionally, in general veterinary practice and particularly in referral institutions, it is common for those dogs being investigated for hypothyroidism to already have received drug therapy of one type or another. Consequently, a clear understanding of the effect that NTI and drug therapy may have on these tests is particularly important when interpreting them in dogs being investigated for suspected hypothyroidism.

Effects of Non-thyroidal Illness on Thyroid Hormones

General Concepts

Several mechanism are responsible for the complicated metabolic alterations in thyroidal physiology that occur in NTI. These include reduction in transport binding protein concentrations and affinity for thyroid hormones, altered peripheral hormone metabolism and production rates, and decreases in peripheral thyroid hormone receptor function. In humans various clinicopathological changes can develop during and following NTI and these have recently been summarised by Beckett & Wilkinson, (1998). The changes include a decrease in circulating TSH during acute illness but an increase, often into the "hypothyroid range", during the recovery phase. Total T_3 concentrations may decrease due to impaired hepatic uptake of T_4 and reduced peripheral conversion of T_4 to T_3. Circulating rT_3 values increase due to impaired clearance and both the concentration of thyroid hormone binding proteins and their affinity for the thyroid hormones may decrease causing

a reduction in circulating total thyroid hormone concentrations. These mechanisms are discussed in more detail below. Some but not all of these features have now also been established in dogs with NTI.

The most commonly recognised clinicopathological thyroidal abnormality in human patients with NTI is the "low T_3 syndrome" in which serum total T_3 concentrations are suppressed whilst total T_4 values remain normal. In more severe, frequently life threatening illnesses, suppression of both serum total T_4 and total T_3 concentrations can occur and is termed the "low T_4 state of medical illness" (Nicoloff & LoPresti, 1996). Previous human and veterinary reports have used the phrase "euthyroid sick syndrome" synonymously with what is strictly the low T_3 syndrome (Wartofsky & Burman, 1982; Ferguson, 1988; Peterson & Ferguson, 1989). However, recently the term "euthyroid sick syndrome" has been more commonly used to describe the general suppressive effect of NTI on the thyroid gland (Ferguson, 1994; Feldman & Nelson, 1996; Nicoloff & LoPresti, 1996).

The Low T_3 Syndrome in Humans

In humans the "low T_3 Syndrome" is the most common thyroidal derangement associated with NTI and occurs in between 25 and 50 % of hospitalised patients (Bermudez, Surks & Oppenheimer, 1975). Both total and absolute free T_3 concentrations are usually depressed whilst total and free T_4 concentrations remain normal. The principal cause of the low T_3 syndrome is related to reduction in the peripheral activity of the 5'-D enzyme. Reduced 5'-D activity causes a reduction in peripheral conversion of T_4 to T_3 and of rT_3 to 3,3'-T_2. Consequently there is a decrease in circulating total T_3 and an increase in total rT_3 concentrations (Wartofsky & Burman, 1982). It is now clear that the increase in rT_3 concentration which occurs in NTI is not due to increased production from T_4, but results solely from the reduced disposal rate (LoPresti, Gray & Nicoloff, 1991). Whilst there is a reduction in the production rate of T_3, the conversion of T_4 to rT_3 remains unaffected, neither significantly increasing or decreasing. However, since total T_4 concentrations do not increase in NTI, there is clearly a discrepancy in T_4 disposal products. Evaluation of other pathways of T_4 disposal suggest that NTI may be associated with an increased production of T_3 sulphates (T_3-S) (Chopra, Wu, Teco & Santini, 1992).

An elevation in rT_3 has been documented in numerous NTI in humans including acute pyrexia, sepsis, advanced human immunodeficiency virus (HIV) infection and chronic cirrhosis (Chopra, Chopra, Smith, Reza & Solomon, 1975; Nicoloff & LoPresti, 1996). This feature has been used to assist in the differentiation of NTI from hypothyroidism since

in hypothyroidism total rT_3 concentrations are generally subnormal (Chopra *et al.*, 1979a). The notable exception is renal disease in which rT_3 may remain normal despite markedly depressed total T_3 concentrations (Kaptein, Feinstein, Nicoloff & Massry, 1983).

The magnitude of depression of total and free T_3, and the associated increase in total and free rT_3 in the low T_3 syndrome has been well correlated with the clinical outcome in humans and therefore has prognostic utility. The time taken for recovery as well as the likelihood of recovery can both be inferred from the severity of the changes. Similarly, the reversal of these abnormalities is typically associated with clinical improvement and a more favourable prognosis (DeGroot, Larsen & Hennemann, 1996).

It is seemingly sensible that during times of illness or physiological stress there is a reduction in total and free T_3 concentrations, the most potent of the thyroid hormones, in an attempt to reduce catabolic activity and thereby conserve energy. However, the basic question of thyroidal status within the tissues themselves in NTI remains largely unanswered. The absence of clinical hypothyroidism in the low T_3 syndrome suggests that tissue euthyroidism is probably maintained although this is rather speculative. Whilst actual tissue thyroid hormone concentrations are reduced, NTI and starvation are also associated with a decrease in the number of nuclear T_3 receptors in both humans and experimental animal models. Easier saturation of the total nuclear receptor load despite reduced hormone concentrations has been suggested as one possible mechanism whereby "relative" euthyroidism is maintained despite reduced thyroid hormone concentrations (DeGroot *et al.*, 1996). Nevertheless this issue remains unclear and following extensive studies on fasting patients, it is generally accepted that NTI is probably associated with a mild form of thyroid deficiency that limits the breakdown of skeletal muscle for gluconeogenesis (Nicoloff & LoPresti, 1996). However, such a functional hypothyroid state is unlikely to be present in all tissues since circulating TSH concentrations do not increase.

The Low T_4 state of Medical Illness in Humans

The low T_4 state of medical illness, whereby total T_4 and total T_3 concentrations are depressed is a less frequent finding in humans with NTI than it is in dogs (Ferguson, 1988) and is certainly less common than the low T_3 syndrome. Classically this condition is characterised by subnormal total but normal free thyroid hormone concentrations although in very severe illnesses absolute free hormone concentrations may also be affected. Typically, humans progress from the low T_3 syndrome to the low T_4 state of medical illness as the severity of their disease increases. This process may take days or weeks if the

progression is gradual, but can occur within hours after major trauma, shock or sepsis (Vitek & Shatney, 1987). Several mechanisms are responsible for the reduction in thyroid hormones which occurs in severe NTI and these are discussed below.

Thyroid hormone binding proteins are frequently subnormal in the low T_4 state of medical illness as a result of impaired synthesis or as a consequence of the acute phase response. The severity and chronicity of the illness will influence the extent of these changes. In humans with NTI, there is also a switch from the hepatic production of normal TBG to a desialated variety of the protein known as "slow TBG" (Beckett & Wilkinson, 1998). This modified protein has an affinity for the thyroid hormones only approximately one tenth that of normal TBG. Since in humans TBG is the major thyroid hormone binding protein this can significantly reduce the total thyroid hormone carrying capacity in NTI.

The presence of inhibitors of thyroid hormone protein binding are important determinants of thyroid hormone concentrations in the low T_4 state of medical illness. In contrast to the low T_3 syndrome in which free T_4 is relatively unaffected, the circulating free T_4 fraction is usually increased in the low T_4 state of medical illness suggesting altered protein binding (Ferguson, 1997). Since the first identification of serum THBI by Chopra *et al.,* (1979b) their presence in up to 90 % of patients with NTI has been confirmed (Chopra, Huang, Beredo, Solomon. & Chua Teco, 1986). These serum THBI cause displacement of hormone from the carrier proteins resulting in an increase in the circulating free hormone fraction. This in turn suppresses pituitary synthesis and release of TSH. The stimulus for thyroid hormone production is consequently reduced and total circulating thyroid hormone concentrations decrease until the free hormone concentration returns to normal. In patients with NTI, free hormone concentrations are therefore usually maintained despite the subnormal total hormone concentrations. It is the THBI that are thought to be responsible for this physiological effect (Oppenheimer, Schwartz, Mariash & Kaiser, 1982). These binding inhibitors may originate from leakage from diseased tissues but an increase in the concentration of "normal" serum constituents has also been proposed as a possible mechanism (Chopra, Solomon, Teco & Eisenberg, 1982) and the binding inhibitors have been shown to have the characteristics of normally occurring unsaturated fatty acids such as oleic acid (Haynes, Lockett, Farmer, Fitch, Bradwell, Sheppard & Ramsden, 1989).

An analytical consequence of elevated THBI concentrations occurs in free hormone assays which require any degree of sample dilution as part of the procedure. Dilution of samples containing THBI causes a reduction in the in vitro THBI concentration. This reduces the inhibitory effects of THBI on hormone protein binding and consequently the in-

vitro free hormone concentration falls. The dialysis of samples can be shown to be equivalent to dilution in this regard. Therefore, dialysis methods for free hormone estimation require that the semi-permeable membrane does not allow transit of THBI between dialysate and dialysand compartments (Ekins, 1985).

In humans, there is a strong correlation between the magnitude of thyroid hormone suppression and the severity of illness and therefore the likelihood of survival (Slag, Morely, Elson, Crowson, Nuttall & Shafer, 1981). Consequently thyroid hormones are used as a non-specific prognostic indicator in patients with NTI and indeed the prognostic value of suppressed circulating thyroid hormone concentrations alone is similar to that of more complex multifactorial prognostic methods such as the Apache II scoring system (Kaufman, Dlott, Townsend, Mizuno, Weiner & Nicoloff, 1988). Similarly, the increase in the free T_4 fraction has also been demonstrated to inversely correlate with the likelihood of surviving (Kaptein, Weiner, Robinson, Wheeler & Nicoloff, 1982). Similar studies have confirmed the correlation of low total T_4 with increased mortality in hospitalised cats (Mooney, Little & Macrae, 1996) and a similar association is expected in the dog (Feldman & Nelson, 1996).

Thyrotropin in Non-thyroidal Illness

Endogenous circulating basal TSH concentration and TSH response to TRH administration are both significantly reduced in humans with severe NTI (Nicoloff & LoPresti, 1996). In contrast, even in the earliest stages of primary hypothyroidism in humans, TSH concentrations increase, as does the TSH response to TRH administration. Since this response is the opposite to that of patients with NTI it has been considered as evidence that tissue euthyroidism is maintained in NTI. However, this is certainly not conclusive evidence since it is known that NTI may suppress the secretion of TSH in patients with concurrent primary hypothyroidism (DeGroot *et al.*, 1996).

Sumita *et al.*, (1994) prospectively evaluated 41 human patients with various critical illnesses of whom ultimately 18 survived and 23 died. Mean ± s.e.m. basal endogenous TSH (μU/ml) concentration was significantly lower in the non-surviving patients (0.23 ± 0.05) compared to the surviving patients (1.35 ± 0.33). Peak TSH concentration after TRH administration was also significantly blunted in the non-survivors (1.42 ± 0.38) compared to the survivors (7.52 ± 1.53). Only five patients had a normal TSH response to TRH, and all of these individuals survived.

An elevation in endogenous circulating glucocorticoids may be at least partly responsible for the reduction in circulating basal TSH values in NTI. Other mechanisms causing suppression may include an elevation in cytokines plus opioidergic, dopaminergic and somatostatinergic pathways although these are currently less well characterised (Scanlon & Toft, 1996). The fall in serum TSH in these patients contributes to the decrease in thyroid hormone concentrations seen during NTI.

Effect of Non-thyroidal Illness on Thyroid Function in Dogs

In dogs, subnormal circulating total T_4 concentrations have been reported in hyperadrenocorticism, diabetes mellitus, hypoadrenocorticism, chronic renal failure, hepatic diseases and a variety of medical illnesses requiring intensive care (Ferguson, 1994). These conditions are typically also associated with reduced circulating total T_3 concentrations (Ferguson, 1988). Despite these studies, the effect of NTI on thyroid function is less well characterised in dogs than it is in humans (Panciera, 1990b). Nevertheless, it appears that certainly in comparison with humans, suppression of both total T_4 and T_3 is more common than is suppression of T_3 alone.

Total Thyroid Hormones

Peterson *et al.,* (1984) evaluated total T_4 and T_3 concentrations in 102 dogs with spontaneous hyperadrenocorticism. Subnormal total T_4 and T_3 concentrations occurred in 58 (56.9 %) and 53 (52.0 %) of the dogs, respectively. Of the 102 cases, total T_4 alone was depressed in 16 (15.7 %), total T_3 alone was depressed in 11 (10.8 %), and in 42 (41.2 %) cases both hormones were subnormal. In 20 cases monitored after therapy for hyperadrenocorticism, mean total T_4 and T_3 concentration increased significantly, and both hormones normalised in all but one of the dogs. In the same study, circulating total T_4 concentration was also determined in another 22 dogs with spontaneous hyperadrenocorticism before and four hours after TSH administration. Both pre and post total T_4 results were significantly depressed compared with those from healthy dogs. However, there was a significant increase in total T_4 after TSH administration, increasing by at least 100 % in all cases. Thyroid gland histopathological examination in 33 of the hyperadrenocorticoid dogs prior to therapy did not show evidence of primary thyroidal disease. The authors therefore concluded that the changes in thyroid hormone concentrations were most likely the result of either glucocorticoid mediated thyrotropin

suppression, altered serum thyroid hormone binding or alterations in peripheral thyroid hormone metabolism.

Ferguson, (1988) reported subnormal total T_4 and T_3 concentrations in dogs with diabetic ketoacidosis and suggested that the severity of the illness and adequacy of therapy were also important factors in this regard. However, the detailed data on these cases were not provided.

Nelson *et al.,* (1991) excluded hypothyroidism in 59 dogs in which hypothyroidism had been a diagnostic consideration, based on the results of bovine TSH response tests. The various NTI reported in these dogs consisted of allergic inhalant dermatitis, flea allergy dermatitis, pruritic superficial folliculitis, hyperadrenocorticism, idiopathic seborrhoea, peripheral neuropathy, food hypersensitivity and idiopathic generalised megaoesophagus. Total T_4 concentration was subnormal in 12 (20 %) of these cases. When categorised based on the type of NTI, circulating total T_4 concentrations were not significantly different between those dogs with hyperadrenocorticism or peripheral neuropathy compared with the hypothyroid dogs but were significantly lower in the hypothyroid group compared with each of the other NTI. Total T_3 concentrations were not significantly different between any of the groups studied.

Paradis *et al.,* (1991) evaluated circulating total T_4 concentrations in 51 euthyroid dogs with various dermatopathies. Hypothyroidism was excluded based on TSH response test results. Subnormal total T_4 concentrations were reported in seven (13.7 %) of the cases. However, the case material used in that study was pre-selected to exclude dogs with serious NTI. Therefore, the incidence of subnormal total T_4 results amongst the original study population, would almost certainly have been considerably greater than that reported.

Larsson, (1988) excluded hypothyroidism in 17 dogs with NTI based on failure to respond to THRT and in most cases categorisation was supported by TSH response test results. Subnormal total T_4 and total T_3 concentrations were reported in 10 (58.8 %) and one (5.9 %) of the cases respectively.

Vail, Panciera & Ogilvie, (1994) evaluated 83 dogs, either with or without neoplastic disease, and with or without chronic weight loss. Mean (± s.d.) total T_4 and total T_3 concentrations were 15.1 (± 4.4) nmol/L and 0.68 (± 0.16) nmol/L in cachexic tumour-bearing dogs, respectively both of which were significantly lower than the corresponding values of 25.8 (± 4.9) nmol/L and 1.12 (± 0.19) nmol/L in healthy control dogs. Cachexic non-tumour-bearing dogs had corresponding values of 16.2 (± 6.2) nmol/L and 0.74 (± 0.28) nmol/L which were also significantly lower than the results from the healthy dogs.

The non-cachexic tumour-bearing dogs had total T_4 and total T_3 concentrations of 23.8 (± 7.7) nmol/L and 1.02 (± 0.22) nmol/L, respectively which were not significantly different to those of the healthy dogs. The authors concluded that the degree of suppression of thyroid function was correlated with either nutritional status or more likely the severity of illness in dogs.

Elliot, King & Zerbe, (1995) evaluated circulating total T_3 and T_4 concentrations before and after bovine TSH administration in 109 dogs admitted to an intensive care facility. Dogs were classified as either hypothyroid or euthyroid based on the TSH response test results. Hypothyroidism was excluded in 67 of the cases and these were subsequently used for the remainder of the study. Decreased total T_4 and total T_3 concentrations occurred in 61 % and 56 % of all euthyroid cases, respectively. Thyroid hormone concentrations were evaluated by comparing results in those dogs which died of their illness (n=20), compared with those which did not die (n=47). Of the dogs that died, 75 % had subnormal (less than laboratory reference range) total T_3 concentration compared to 49 % of those that survived. There was a direct correlation between the suppression of total T_3 and likelihood of mortality. Neither pre nor post TSH total T_4 concentration were significantly different between the survivors and non-survivors. However, both pre and post-TSH total T_3 concentrations were significantly decreased in the dogs that died (0.99 ± 0.26 and 1.48 ± 0.59 nmol/L, respectively) compared to the survivors (1.54 ± 1.24 and 2.2 ± 1.01 nmol/L, respectively). The authors concluded that depressed total circulating T_4 is the most common thyroidal derangement associated with life-threatening critical illness, but that the change in circulating T_3 was more closely correlated with eventual outcome.

Peterson *et al.,* (1997) investigated 108 dogs for possible hypothyroidism, and excluded the diagnosis in 54 of them based on the results of TSH response tests. Depressed serum total T_4 concentrations were reported in 10 (18.5 %) of the dogs. Circulating total T_3 was measured in 38 of these cases and was below the reference range in three (7.9 %) of them.

Scott-Moncrieff *et al.,* (1998) evaluated 49 dogs for hypothyroidism and based on TSH response tests excluded this diagnosis in 33 of them. The specificity of circulating total T_4 estimation for hypothyroidism was 0.78. However, the individual thyroid hormone data were not provided.

Panciera, Helfand & Soergel, (1995) evaluated the effect of administration of interleukin-2, an important mediator of inflammatory disease, to four adult dogs on total T_4 and total T_3 concentration. There was a dramatic and rapid decrease in both thyroid

hormones during administration of interleukin-2, with total T_4 decreasing by 50-75 %, and total T_3 decreasing by 70-80 % in all dogs. This model of NTI mimicked the low T_4 state of medical illness encountered in dogs with spontaneous NTI, and demonstrates the potentially profound effect that NTI may exert on thyroid hormone metabolism.

Several studies have examined the effect of NTI on serum total rT_3 in the dog but have produced conflicting results. Larsson, (1987) reported elevated circulating total rT_3 concentrations in dogs with hepatic and renal disease, and diabetes mellitus but not hyperadrenocorticism which is similar to the changes which usually occur in humans. However, studies of fasted dogs have reported either no change, or even a slight decrease in rT_3 concentrations (de Bruijne, Altszuler, Hampshire, Visser & Hackeng, 1981; Laurberg & Boye, 1984). In the report by Ferguson & Peterson, (1992) reviewed in more detail below, rT_3 was measured in 42 dogs with hyperadrenocorticism and compared to results from 103 clinically healthy dogs. Mean rT_3 concentration was significantly lower in the dogs with hyperadrenocorticism (0.17 nmol/L) compared to the healthy dogs (0.39 nmol/L) with 48 % of the hyperadrenocorticoid dogs having subnormal values. The variation in rT_3 results between studies may reflect differences between the thyroidal effects of glucocorticoid excess typical of hyperadrenocorticism and the other metabolic abnormalities such as 5'-D activity and protein binding inhibition encountered in various other NTI.

Free Thyroid Hormones

The various methodologies available for free T_4 estimation and their relative limitations have already been reviewed in Chapter 4. Only methods considered as genuine free hormone methods will be reviewed.

Ferguson, (1988) reported elevation in the free T_4 fraction measured by equilibrium dialysis in 10 of 45 (22 %) dogs with spontaneous hyperadrenocorticism suggesting diminished serum protein binding. However, 13 (29 %) of the cases had subnormal absolute circulating free T_4 results indicating that either reduced thyroidal hormonal secretion, most likely mediated via cTSH suppression, or enhanced thyroid hormone metabolism also contributed to the thyroidal changes. In the same report, the effect of experimental removal of renal tissue reducing glomerular filtration rate to approximately 20 % of the pre-surgery values with the consequent induction of severe uraemia, on free T_4 concentration was evaluated. The free T_4 fraction was reported to approximately double after the surgery whilst total T_4 values decreased by approximately half. Consequently, the

absolute free T_4 concentrations remained approximately normal although the actual data were not provided.

Ferguson & Peterson, (1992) evaluated a free T_4 method which was a modification of the indirect dialysis technique reported by Sterling & Brenner, (1966) in 42 dogs with hyperadrenocorticism and in 103 clinically healthy dogs. Broadly speaking, the results were similar to those reported previously by Ferguson, (1988). The mean free T_4 fraction was significantly greater in the hyperadrenocorticoid dogs (0.15 ± 0.018 %) compared with the healthy animals (0.09 ± 0.003 %) indicative of reduced protein binding. An increased free T_4 fraction, was present in 16 (38 %) of the hyperadrenocorticoid dogs. Despite this, the mean free T_4 concentration was significantly lower in the dogs with hyperadrenocorticism (16.9 ± 2.3 pmol/L) compared with the healthy dogs (23.6 ± 1.0 pmol/L). Although reduced protein binding was present in 16 cases, the total T_4 concentration was within the reference range in seven of these dogs. Reduced peripheral conversion of T_4 to T_3 which commonly occurs in human patients with hyperadrenocorticism did not appear to occur to any great extent since few dogs had subnormal T_3 to free T_4 ratios. The authors concluded that the mechanism responsible for the changes in free T_4 concentrations which occur in hyperadrenocorticism differed between humans and dogs. Reduced pituitary cTSH secretion was suggested as a possible cause of the thyroidal derangements in canine hyperadrenocorticism. This was supported by the failure of rT_3 to increase as indicated previously.

Scott-Moncrieff *et al.,* (1994) determined free T_4 using a modified equilibrium dialysis kit in 74 dogs. These cases included healthy dogs, hypothyroid dogs and cases with a variety of NTI including hyperadrenocorticism, obesity, hypoalbuminaemia and megaoesophagus. Cases were classified based on clinical findings, routine diagnostic testing including TSH response testing, and the requirement for THRT. Mean (± s.d.) free T_4 was significantly decreased in the hyperadrenocorticoid (18.02 ± 7.7 pmol/L) and hypothyroid dogs (2.6 ± 2.6 pmol/L) compared with the healthy animals (25.7 ± 10.3 pmol/L). Free T_4 was not significantly different between the other groups of illnesses and the healthy cases. The authors provided no explanation to suggest why the free T_4 results were affected by hyperadrenocorticism but not by the other illnesses and no data regarding the severity or duration of the conditions were provided.

Peterson *et al.,* (1997) evaluated 54 euthyroid dogs in which clinical signs suggestive of hypothyroidism had been present but in which that diagnosis was excluded based on TSH response test results. Circulating free T_4 was determined from these and

from 150 clinically healthy dogs using the same modified equilibrium dialysis kit as used by Scott Moncrieff *et al.,* (1994). A free T_4 reference range was determined as the 5th to 95th percentiles of the healthy dogs' results. Median free T_4 concentration was 20 pmol/L in the dogs with NTI compared to 22 pmol/L in the healthy dogs and 2 pmol/L in the hypothyroid dogs. Four (7 %) of the dogs with NTI had a subnormal free T_4 result compared with 53 of the 54 (98 %) hypothyroid dogs.

Thyroglobulin autoantibodies

A detailed review of the estimation of circulating TgAb in canine NTI has already been provided in Chapter 7. Those points will not be reiterated here. However, until the recent report by Nachreiner *et al.,* (1998) none of the studies of canine TgAb estimation utilised an assay which was considered adequately reliable. Consequently the apparently high prevalence of positive TgAb results from dogs with NTI was difficult to interpret. With the development of the assay reported by Nachreiner *et al.,* (1998), that technical difficulty has apparently now been largely overcome, and in that study positive TgAb results were identified in 3.4 % of 146 dogs with NTI including dogs with calcium disorders, hyperadrenocorticism, hypoadrenocorticism, diabetes mellitus and obesity. However, the selection of these limited categories of illnesses may not represent cases with other more wide ranging diseases. In particular, the selection of dogs with hypoparathyroidism, hypoadrenocorticism and diabetes mellitus may increase the likelihood of including dogs with immune mediated endocrine disorders which are therefore prone to autoimmune thyroiditis (Dixon, 1998). The true prevalence of TgAb in dogs with truly wide ranging NTI therefore remains unclear.

Thyroid Hormone Therapy in the Treatment of Non-thyroidal Illness

Despite, as discussed above, the absence of a definitive answer to the question "is tissue euthyroidism maintained in the face of NTI ?", there is convincing evidence that thyroid hormone replacement in patients with NTI is at best of no benefit and in fact may increase mortality. As already indicated in Chapter 6, Brent & Hershman, (1986) studied the effect of intravenous T_4 replacement therapy on mortality in hospitalised human patients with severe NTI and did not identify any effect of such therapy on eventual outcome. Little, (1985) evaluated the effect of T_4 therapy in male rats with decreased total and free T_4 concentrations as a consequence of experimental infection with *Streptococcus pneumoniae.*

Those cases treated with T_4 were significantly more likely to die and had a significantly decreased time to death compared to the control animals. The author concluded that the decrease in circulating thyroid hormone concentrations in NTI is beneficial and may serve a role in maintaining homeostasis by conserving energy. Studies on the effect of THRT in dogs with severe NTI and subnormal thyroid hormone concentrations have not been reported.

Effects of Drug Therapy on Thyroid Hormones

General Comments

The mechanisms by which pharmacologic agents alter thyroid hormone metabolism are varied but include changes in thyroid hormone secretion, protein binding, intracellular metabolism and peripheral conversion of T_4 to T_3 and rT_3 (Meier & Burger, 1996). Numerous categories of drugs used in veterinary practice potentially interfere with thyroid hormone metabolism. These include glucocorticoids, anticonvulsants, quinidine, non-steroidal antiinflammatory drugs, sulpha-containing antimicrobials and iodine-containing radiocontrast agents (Ferguson, 1994). However, in practice the most important of these are glucocorticoids and sulpha-containing antibiotics since they are frequently used in dermatological cases, a common clinical feature associated with hypothyroidism.

Glucocorticoids

In human patients receiving glucocorticoid therapy, a number of thyroidal abnormalities may occur. Broadly speaking, the effect of therapy is greatest on total T_3 concentrations but in some patients, total T_4 or free thyroid hormone concentrations may also decrease (Rosychuk, 1982). Glucocorticoids decrease TBG and TBPA production in humans which causes a reduction in the total circulating thyroid hormone pool. This primarily affects total T_4 concentrations. There is also a direct suppressive effect on pituitary TSH production which contributes to the reduction in total T_4 (Wilber & Utiger, 1969; Nicoloff, Fisher & Appleman, 1970). A similar effect has recently been reported in dogs (Dixon & Mooney, 1997). A transient 'rebound' period during which TSH is temporarily elevated after the cessation of glucocorticoid therapy is also recognised in humans and may last for up to a week after stopping the treatment (Wilber & Utiger, 1969; Nicoloff *et al.,* 1970; Spencer *et al.,* 1987). However, similar studies in the dog are lacking and currently the influence that this phenomenon may have on the interpretation of endogenous cTSH in the diagnosis of

canine hypothyroidism is unclear. Glucocorticoids also inhibit the peripheral conversion of T_4 to T_3 and this can have a marked effect on circulating total T_3 concentrations. Indeed, exogenous glucocorticoid therapy can reduce serum total T_3 values by up to 40 % (Gambert, 1996).

Woltz, Thompson, Kemppainen, Munnell & Lorenz, (1983) demonstrated that the intramuscular administration of 2.2 mg/kg body weight prednisone given every other day to five healthy dogs significantly decreased plasma T_3 within eight hours of therapy although total T_4 did not significantly decrease until days nine and 11 of treatment. Kemppainen, *et al.,* (1983) administered 2.2 mg/kg body weight prednisone intramuscularly on alternate days to 10 healthy adult crossbreed dogs for 21 days. Thyroid function was evaluated throughout the experiment by measurement of resting total T_4 and total T_3 concentrations, and total T_4 response to both TSH and TRH administration. Mean ± s.d. resting total T_4 and total T_3 concentrations were significantly decreased in the treated dogs (9.3 ± 2.4 nmol/L and 0.29 ± 0.04 nmol/L, respectively) compared with control dogs (39.5 ± 5.9 nmol/L and 0.58 ± 0.06 nmol/L, respectively). The dogs given prednisone had significantly reduced total T_4 and total T_3 concentrations (23.8 ± 3.9 nmol/L and 0.37 ± 0.03 nmol/L respectively) four hours after intravenous administration of 250 µg TRH compared with the control dogs (55.5 ± 6.0 nmol/L and 0.87 ± 0.1 nmol/L, respectively). However, results were not significantly different between the treated and untreated dogs four hours after intravenous administration of 0.4 i.u./kg body weight exogenous TSH. Laurberg & Boye, (1984) administered a single dose of 12 mg dexamethasone to 12 healthy mixed breed dogs weighing between 18 and 24 kg. The route of drug administration was not indicated. There was no significant effect on total T_4 concentrations within 48 hours after drug administration. A transient decrease in total T_3 values occurred which was statistically significant at 24 hours but which had returned to near pre-treatment values by 48 hours. There was also a marked and statistically significant increase in total rT_3 concentration after the drug administration which lasted for more than 48 hours. Torres *et al.,* (1991) confirmed that basal total T_4 and total T_3 concentration, and total T_4 response to TSH administration were significantly decreased within 24 hours of oral administration of 1.1 mg/kg body weight prednisolone twice daily to 12 healthy beagle dogs. However, the same drug used orally at 0.55 mg/kg body weight twice daily for a month did not significantly alter serum total T_4 concentration although it did significantly reduce total T_3 values (Moore, Ferguson & Hoenig, 1993).

It is clear from these studies that the steroid type, dose, length of therapy and route of administration can profoundly influence the magnitude and duration of these effects in both humans and dogs.

Sulphonamide Antibiotics

Panciera & Post, (1992) evaluated the effect of sulphadiazine-containing antibiotics at a therapeutic dose rate (12.5 mg/kg) on circulating thyroid hormones in six clinically healthy dogs. There was no apparent effect of the medication on mean total T_4, total T_3 or free T_4 concentration at any time during the study period. Neither was there any effect of the drug on total T_4 response to TSH administration.

Hall *et al.*, (1993) evaluated the effect of administration of sulphamethoxazole-containing antibiotics at a standard therapeutic dose (30mg/kd twice daily) to 21 dogs with pyoderma and normal baseline thyroid hormone concentrations. Six weeks after starting therapy, there was a significant suppression of mean basal total T_4 concentration. In three dogs a TSH response test was performed and in each case there was a reduction in post-TSH total T_4 concentration was reported although the data were not provided. The serum total T_3 concentrations were reduced in 13 of the 21 dogs six weeks after starting therapy but the reduction was not statistically significant in the group as a whole.

Using the same drug and dosing regime as Hall *et al.*, (1993), Campbell *et al.*, (1996) demonstrated suppression of both total T_4 and total T_3 and elevations in cTSH concentrations in 12 clinically healthy dogs within three weeks of starting therapy. The cTSH values returned to normal three weeks after the cessation of treatment. Total rT_3 concentrations were also reduced by therapy, suggesting that the effects on thyroid hormone concentrations most likely resulted from direct thyroidal suppression. The authors concluded that the drug can cause a temporary iatrogenic hypothyroidism and has the ability to produce confusing diagnostic test results.

Torres, McKeever & Johnston, (1996) reported the development of clinical signs and biochemical abnormalities consistent with hypothyroidism in a dog receiving sulphadiazine therapy at approximately 50 mg/kg for 18 weeks. Severe suppression of baseline thyroid hormone concentrations, and a complete absence of response to exogenous TSH administration was reported which resolved within seven days of cessation of therapy.

Gookin, Trepanier & Bunch, (1999) reported the development of iatrogenic clinical hypothyroidism in a dog as a result of chronic trimethoprim-sulphadiazine therapy.

However, the true role of this drug in that case was complicated by the concurrent administration of exogenous THRT.

Hall *et al.,* (1993) investigated the mechanism of action of sulphonamides on thyroid hormone metabolism by radionuclide imaging and suggested it to be interference with iodide metabolism causing failure of thyroid hormone synthesis. The mechanism has been reported to be inhibition of TPO activity, an enzyme integral to several of the production steps in thyroid hormone synthesis (Gupta, Eggo, Uetrecht, Cribb, Daneman, Reider, Shear, Cannon & Spielberg, 1992; Doerge & Decker 1994). Clearly, based on the above studies, there is variation in the effect of sulphonamide preparations depending on both the preparation and dose used and the duration of therapy.

Phenobarbital Drugs

Gaskill, Burton, Gelens, Ihle, Miller, Shaw, Brimacombe & Cribb, (1999) evaluated serum total T_4 and cTSH concentrations from 126 dogs with a provisional diagnosis of epilepsy. Seventy eight of the dogs were being medicated with phenobarbital whilst 48 were not. Mean total T_4 was significantly decreased and mean cTSH results were significantly increased in the treated compared with the untreated dogs. However, the increase in cTSH concentration resulted in a mean value (0.22 ng/ml) which remained well within the reference range. Dogs who had suffered seizure activity within the 24 hours prior to blood testing had significantly depressed total T_4 concentrations compared to those which had not. Kantrowitz, Peterson, Trepanier, Melian & Nichols, (1999) also evaluated the effect of phenobarbital administration on indices of thyroid function in 78 dogs with seizure disorders compared with 150 clinically healthy dogs receiving no medication. Of the seizure disorder cases, 55 were receiving phenobarbital alone, 15 received phenobarbital and bromide, and eight were treated with bromide alone. Total and free T_4 (measured by equilibrium dialysis) concentrations were significantly lower in the dogs receiving phenobarbital compared with the healthy dogs. There was no significant difference in mean total T_3 or cTSH concentrations between the two groups. One possible explanation for the difference in cTSH results reported between these two studies, is that in the report of Gaskill *et al.,* (1999) the dogs who were not receiving phenobarbital were epileptic, and many (66 %) had had seizure activity within the previous 24 hours. This may have reduced the cTSH concentration within these "control" dogs. In contrast the unmedicated dogs studied by Kantrowitz *et al.,* (1999) were clinically healthy. The difference in results may therefore be partly related to the difference in the "control" groups used.

8.2 INTRODUCTION

The effect of NTI and commonly used drug therapies on tests of thyroid function is poorly understood in dogs compared to humans. Many of the reports evaluating thyroid function in dogs with NTI have principally studied the effect of hyperadrenocorticism on these tests, which is not necessarily representative of the effects of other NTI. Most recent studies of thyroid function tests in dogs have been principally concerned with those cases in which hypothyroidism was a diagnostic consideration. However, little information exists on the performance of these tests in various other types of NTI. Specifically, very limited data has been reported on the effect of various NTI on the more recently released kits for cTSH, free T_4 by dialysis and TgAb estimation in dogs. In contrast to humans and cats, little is known about the role of thyroid hormone estimations as prognostic markers in dogs with NTI. The objective of this study was to investigate a number of clinical parameters and a comprehensive range of thyroid function-related tests in dogs with varying NTI, to identify clinical and endocrine markers of prognostic value in dogs with NTI.

8.3 MATERIALS AND METHODS

Case Material and Sample Collection

A random selection of canine serum and plasma samples submitted to the diagnostic biochemistry and haematology laboratory for routine analysis were used for the study. Samples had been collected as part of the routine investigation of clinical cases at the DVCS. Samples used in the study therefore included those taken from cases suffering from a broad range of disease types and severity. Once aliquots of the samples had been removed for their primary analysis, the remainder was frozen and stored as described in Chapter 2. Breeds were categorised in the same manner as Chapter 5.

Data Storage and Handling

Case details corresponding to each of the samples were collected by conducting multiple interviews with the relevant clinicians in the DVCS. Data were manually recorded to hard copy at the time of interview and subsequently transferred to a computerised database (Microsoft Access for Windows 95, version 7.0, Microsoft Corporation) to facilitate permanent storage and data analysis. An example of a completed form is shown in Appendix 5. The data recorded included reference, signalment, clinical and historical features including the recent known drug therapies in each case prior to sampling. The fields containing data for disease category, disease course, diagnostic method and outcome

had a limited number of options from which clinicians were requested to select only one. These categories are detailed in Tables 27 to 30. If more than one disease category applied to a particular case, the clinician was instructed to select the one which was considered to be the principal complaint. In cases in which multiple diagnostic methods were employed to establish the diagnosis, the method producing the most definitive information was selected.

Each case was assigned a clinical disease score (CDS) corresponding to each sample by the relevant clinician. The CDS was a number between one and 10, chosen by the clinician to indicate his or her opinion of the "metabolic severity" of the dogs illness at the time the sample was collected. A value of one indicated a "normal" dog and a value of 10 corresponded to a dog which the clinician considered "on point of natural death due to profound disease". Clinicians were requested to use a linear scale between these limits to assign the CDS corresponding to each sample. The eventual outcome of cases were usually recorded weeks or months after sample collection by repeated interview with the relevant clinician or by referral to case notes.

For the purposes of this study, drugs capable of suppressing thyroid function were considered to be NSAID's, anticonvulsants, steroids, potentiated sulphonamides and THRT.

Sample Analysis

All samples were analysed for total T_4, free T_4, total T_3, cTSH and TgAb as described in Chapter 2. It was not possible to measure each analyte in every case. Samples with a result less than the assay LoD (Chapter 3) were assigned a value equal to that LoD.

Data Analysis

The ages of the dogs in each of the outcome groups were compared using analysis of variance techniques with post hoc pairwise comparison of means using Newman-Keuls multiple range tests. The effect of thyroid suppressive medication on hormone results was evaluated with Mann-Whitney U tests. The effect of disease category and duration of illness on eventual outcome was evaluated by chi squared analysis. In any analysis in which chi squared testing was performed, only categories which had data cells from more than one variable, with five or more cases in each cell were included in the statistical analysis. In the case of dogs from which samples were obtained on more than one occasion, analyses were only performed using the first sample received from that animal. DPR analysis of the CDS results was performed as described in Chapter 2. Hormone results were considered

abnormal if they were below (total T_4, free T_4 and total T_3) or above (cTSH) the diagnostic limits identified in Chapter 4 otherwise they were considered "normal".

Disease Category
Cardiovascular
Congenital
Dermatological
Endocrine
Gastrointestinal
General Medicine
Immune Mediated
Infectious
Neoplastic
Neurological
Ophthalmological
Orthopaedic
Other
Respiratory
Soft Tissue
Trauma
Urogenital
Unknown

Table 27. Disease categories from which clinicians selected the grouping which in their opinion best represented the primary disease.

Disease Course
Acute
Chronic

Table 28. Disease course options from which clinicians selected the grouping which in their opinion best represented the duration of the primary disease.

Diagnostic Method
Clinical
Clinicopathology
Histopathology
Imaging
Other
Post mortem examination

Table 29. Diagnostic methods from which clinicians selected the technique which in their opinion provided the most definitive diagnostic data.

Outcome	Description
Died	The dog died during the hospitalisation period.
Euthanatised	The dog was euthanatised, or the clinician's recommendation to the owner was that the dog be euthanatised, as a result of the primary complaint.
Survived-partial recovery	The dog survived and was discharged. However, the disease was not cured and/or required permanent medication to control the clinical signs.
Survived-full recovery	The dog survived and was discharged. A full clinical recovery occurred without the need for continuing therapy.

Table 30. Classification of patient outcomes and their corresponding definitions.

8.3 RESULTS

Clinical Material

A total of 233 samples were collected from 205 dogs. Twenty one, six and one dogs had two, three and four samples collected, respectively. The mean ± s.d. age (range) of all the dogs studied was 6.8 ± 4.0 (0.3 to 16.5) years. There were 87 female (46 neutered) and 118 male (24 neutered) dogs. The most commonly represented breeds and breed-types are summarised in Table 31.

Breed	Number of cases
Retriever	41
Spaniel	24
Terrier	23
Collie	20
German shepherd dog	15
Crossbreed	15
Boxer	6
Bullmastiff	5
Rottweiler	5
German shorthaired pointer	4
Akita	3
Bernese mountain dog	3
Great Dane	3
Lurcher	3
Poodle	3
Setter	3
St Bernard	3
Other	26
Total	205

Table 31. Distribution of the most common breeds and breed types investigated. The "other" category corresponds to 20 other breeds each represented by either one or two individuals.

The number of cases in each of the disease categories and their eventual outcome are detailed in Table 32.

Disease Category	Cases	Died	Euthanatised	Partial Recovery	Full Recovery
Cardiovascular	11 (5.4 %)	0	1	8	2
Congenital	2 (1.0 %)	0	1	0	1
Dermatological	5 (2.4 %)	0	0	5	0
Endocrine	8 (3.9 %)	0	1	6	1
Gastrointestinal	24 (11.7 %)	0	3	9	12
General Medicine	13 (6.3 %)	0	3	4	6
Immune Mediated	9 (4.4 %)	0	0	5	4
Infectious	2 (1.0 %)	0	0	1	1
Neoplastic	47 (22.9 %)	0	23	16	8
Neurological	16 (7.8 %)	0	3	10	3
Ophthalmological	11 (5.4 %)	0	0	4	7
Orthopaedic	29 (14.1 %)	1	1	8	19
Other	4 (1.9 %)	0	0	0	4
Respiratory	9 (4.4 %)	0	2	6	1
Soft Tissue	2 (1.0 %)	0	0	1	1
Trauma	6 (2.9 %)	0	0	1	5
Urogenital	5 (2.4 %)	0	1	2	2
Unknown	2 (1.0 %)	0	0	1	1
Total	205	1	39	87	78

Table 32. Classification of cases by principal disease category and eventual outcome.

The principal diagnostic methods used to confirm the diagnosis in each case are outlined in Table 33. The diagnostic modalities which were most commonly used to confirm the diagnosis were clinical opinion, clinicopathological data, histopathological data and imaging techniques, each of which were of similar diagnostic utility.

Only one of 205 dogs died during the study. This case unexpectedly died whilst anaesthetised during surgery for orthopaedic disease, and was not expected to have died as a consequence of the primary complaint. Of the 39 cases which were euthanatised, it was considered likely that the majority of them would have ultimately died of their primary disease if euthanasia had not been performed.

Diagnostic Method	Cases
Clinical	45 (21.9 %)
Clinicopathological	51 (24.9 %)
Histopathological	39 (19.0 %)
Imaging	63 (30.7 %)
Post mortem	6 (2.9 %)
Other	1 (0.5 %)
Total	205

Table 33. Classification of cases by the diagnostic method which was most heavily relied upon to achieve the final diagnosis.

Only neoplasia, gastrointestinal and orthopaedic disease categories had sufficient cases to evaluate any effect of disease category on eventual outcome. Dogs with neoplasia were significantly less likely to make a full recovery but significantly more likely to make a partial recovery compared with the other groups. Orthopaedic cases were significantly more likely to make a full recovery and less likely to make a partial recovery. Analysis of the likelihood of euthanasia depending on disease category was not possible, since only one disease category (neoplasia) had a sufficient number of cases which were euthanatised to allow statistical analysis. However, approximately half of the cases in this group were

euthanatised which is markedly more than in other groupings. Chi squared analysis of the neoplasia group compared to all other dogs when considered as a single group, revealed a highly significant ($p<0.0001$) increase in the likelihood of euthanasia in the dogs with neoplasia compared with those with non-neoplastic illness.

Classification of individual cases by disease course and details of their respective outcomes are summarised in Table 34.

Disease Course	Died	Euthanatised	Partial Recovery	Full Recovery	Total
Acute	0	15	9	19	43
Chronic	1	24	78	59	162
Total	1	39	87	78	205

Table 34. Number of dogs in each outcome category classified by disease course.

There was a significant difference in the outcome of cases depending upon the duration of illness, with acute cases being significantly more likely to be euthanatised but significantly less likely to make a partial recovery than chronic cases which were significantly less likely to be euthanatised.

The CDS results categorised by eventual outcome are detailed in Table 35. and displayed in Figure 26. Statistical analysis of the CDS result from the "died" category was not performed. The CDS was significantly different between each of the remaining three outcome groups. DPR analysis identified an optimal CDS cut-off value of 6.0 for the prediction of euthanasia. This value was associated with a sensitivity, specificity and DPR of 0.67, 0.86 and 0.53, respectively for predicting euthanasia. The mean ($\pm$ s.d.) CDS was significantly increased in the acutely diseased patients (5.1 ± 2.7) compared with the chronically ill patients (3.4 ± 2.2).

Clinical Disease Score (CDS)	Died	Euthanatised	Partial Recovery	Full Recovery	Total
1-2	0	4	34	45	83
3-4	1	6	23	21	51
5-6	0	7	20	8	35
7-8	0	15	10	4	29
9-10	0	7	0	0	7
Mean ± s.d.	3.0 ± 0	6.36 ± 2.55	3.63 ± 1.98	2.67 ± 1.70	3.78 ± 2.39

Table 35. Number of dogs in each outcome category categorised by clinical disease score (CDS) groupings. A CDS value of one indicates a "normal" healthy dog and a value of 10 corresponds to a dog "on point of death due to profound disease". A linear scale is interpolated between these values.

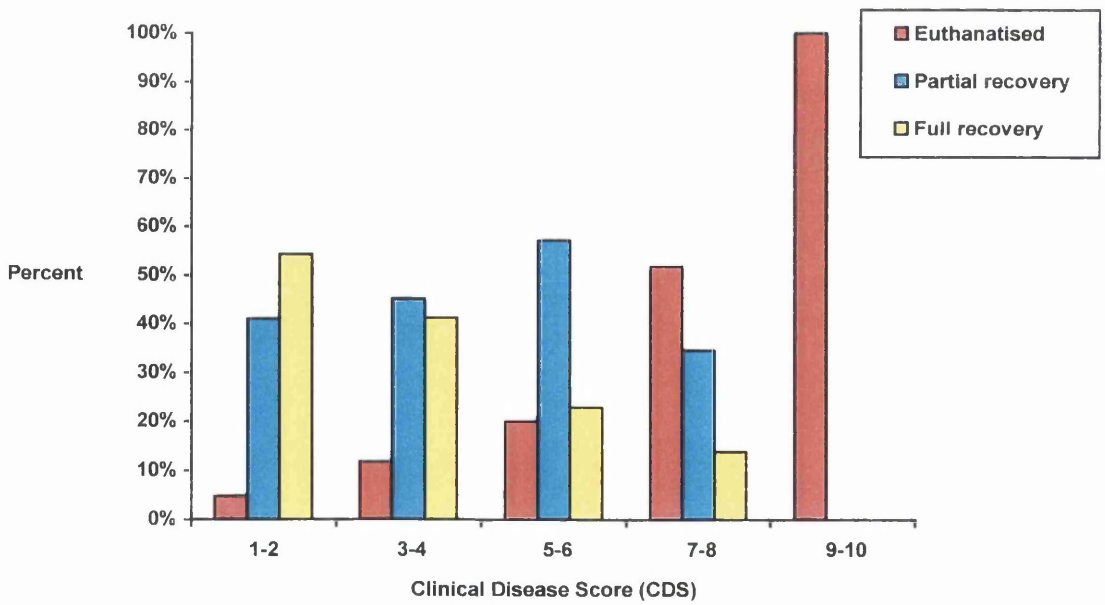


Figure 26. Clinical disease scores (CDS) for 205 dogs categorised by eventual outcome. The columns in each CDS banding total 100 %. A CDS value of one indicates a "normal" healthy dog and a value of 10 corresponds to a dog "on point of death due to profound disease". A linear scale is interpolated between these values.

The outcome of cases categorised by age and gender is summarised in Table 36. Cases which made a full recovery were significantly younger than both those which were euthanatised and those which made a partial recovery. There was no significant difference in age between those dogs which were euthanatised and those which made a partial recovery. There was no significant difference in outcome of dogs based on either gender or neutering status.

Outcome	Age (mean ± s.d.)	Male entire	Male neutered	Female entire	Female neutered
Died	9.42 ± 0	0	0	0	1
Euthanatised	7.66 ± 3.57	19	4	7	9
Partial Recovery	7.51 ± 3.70	39	9	18	21
Full Recovery	5.64 ± 4.33	36	11	16	15
All Cases	6.84 ± 4.02	94	24	41	46

Table 36. Age and gender distribution of cases categorised by eventual outcome.

Thyroid Hormones

The results of all analyses are detailed in Appendix 36. Of the first samples collected from each of the 205 cases, six were collected from dogs which were receiving or recently had received THRT. These six samples were excluded from all further analyses of all thyroid hormone parameters. Of the remaining 199 samples, 62 (31.2 %) were collected from dogs that had not recently received any medication at all. A total of 68 (34.2 %) samples were collected from dogs which had recently received medications capable of suppressing thyroid function. The remaining 69 (34.7 %) samples were collected from dogs which had received other medications. There was no significant difference in eventual outcome of cases when those which had received any type of thyroid suppressive medication were compared with those which had not received these drugs. There was no significant difference in outcome of cases when those which had received some form of therapy were compared with those which had not received any type of medication. Results of analysis of

total T_4, free T_4, total T_3 and cTSH are summarised in Table 37. Combined results of all analyses are presented both before and after exclusion of cases which had recently received thyroid suppressive therapies.

	Dogs receiving thyroid suppressive therapies	Dogs not receiving thyroid suppressive therapies	Combined results
Total T_4 (nmol/L)	16.93 (17.04)	20.65 (15.47)	19.97 (15.90)
Free T_4 (pmol/L)	19.52 (13.14)	22.75 (11.79)	21.55 (12.80)
Total T_3 (nmol/L)	1.26 (0.58)	1.39 (0.77)	1.35 (0.74)
cTSH (ng/ml)	0.12 (0.20)	0.13 (0.22)	0.12 (0.21)

Table 37. Median (semi-interquartile range (SIR)) circulating total T_4, free T_4, total T_3 and cTSH concentration from 199 dogs with various diseases. Results are shown for dogs receiving thyroid suppressive therapies, for dogs not receiving thyroid suppressive therapies and for both groups of dogs combined.

Total T_4 and free T_4 concentrations were both significantly decreased in dogs receiving thyroid suppressive therapy compared to dogs which were not receiving such therapies. There was no effect of these drugs on circulating total T_3 or cTSH concentrations.

Results of total T_4, free T_4, total T_3 and cTSH analyses categorised according to eventual case outcome are summarised in Table 38. Dogs which were euthanatised had significantly decreased total T_4, free T_4 and total T_3 concentrations compared to those which made a full recovery and significantly decreased total T_3 concentrations compared to those which made a partial recovery. No other hormone parameters were significantly different between dogs with different outcomes. The data for total T_4, free T_4 and total T_3 categorised by outcome are displayed in Figures 27 to 29.

	Full Recovery	Partial Recovery	Euthanatised
Total T_4 (nmol/L)	22.46 (13.49)[1]	18.11 (19.34)	12.65 (14.58)[2]
Free T_4 (pmol/L)	24.40 (12.04)[1]	20.61 (11.44)	18.42 (15.65)[2]
Total T_3 (nmol/L)	1.45 (0.76)[1]	1.40 (0.81)[1]	0.99 (0.50)[2]
cTSH (ng/ml)	0.09 (0.20)	0.15 (0.23)	0.11 (0.25)

Table 38. Median (semi-interquartile range (SIR)) circulating total T_4, free T_4, total T_3 and cTSH concentrations in 198 dogs with various illnesses, categorised by their eventual outcome. Hormone results with a different superscript are statistically different from each other.

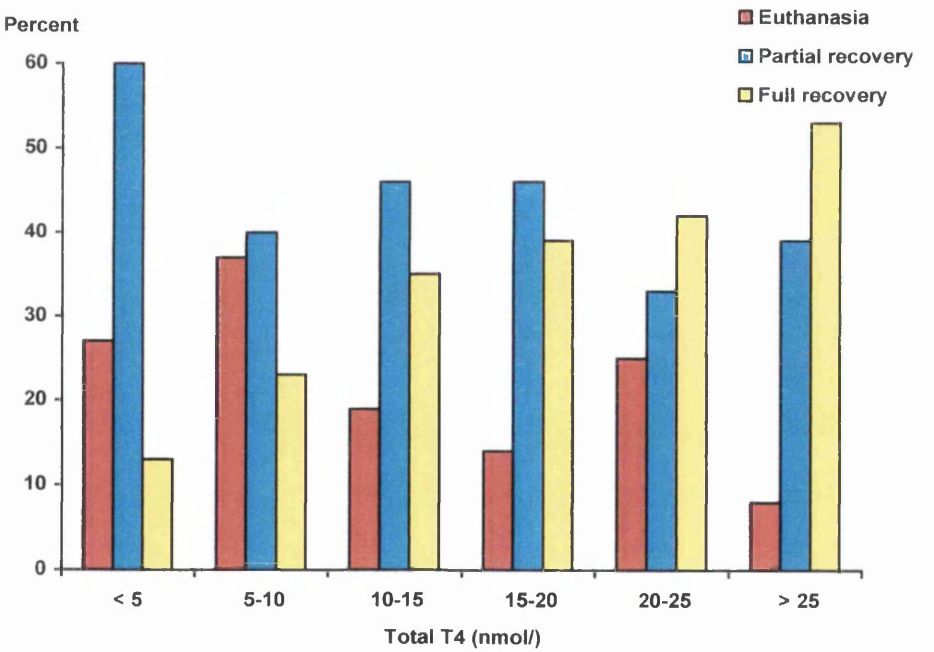


Figure 27. Circulating total T_4 concentrations in dogs with various diseases, categorised by eventual outcome. Results are grouped in 5 nmol/L bandings. The columns in each banding total 100 %.

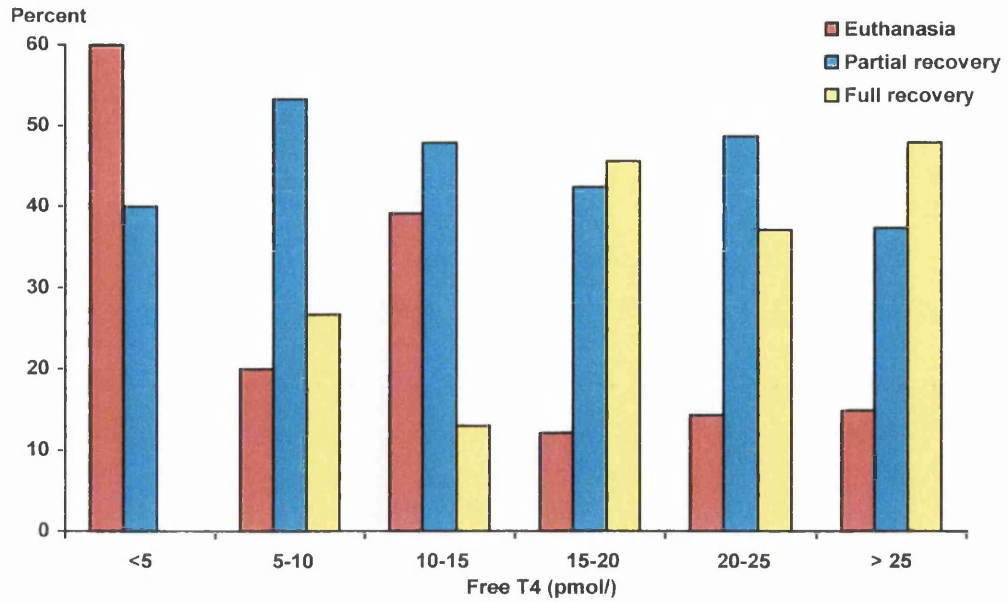


Figure 28. Circulating free T_4 concentrations in dogs with various diseases, categorised by eventual outcome. Results are grouped in 5 pmol/L bandings. The columns in each banding total 100 %.

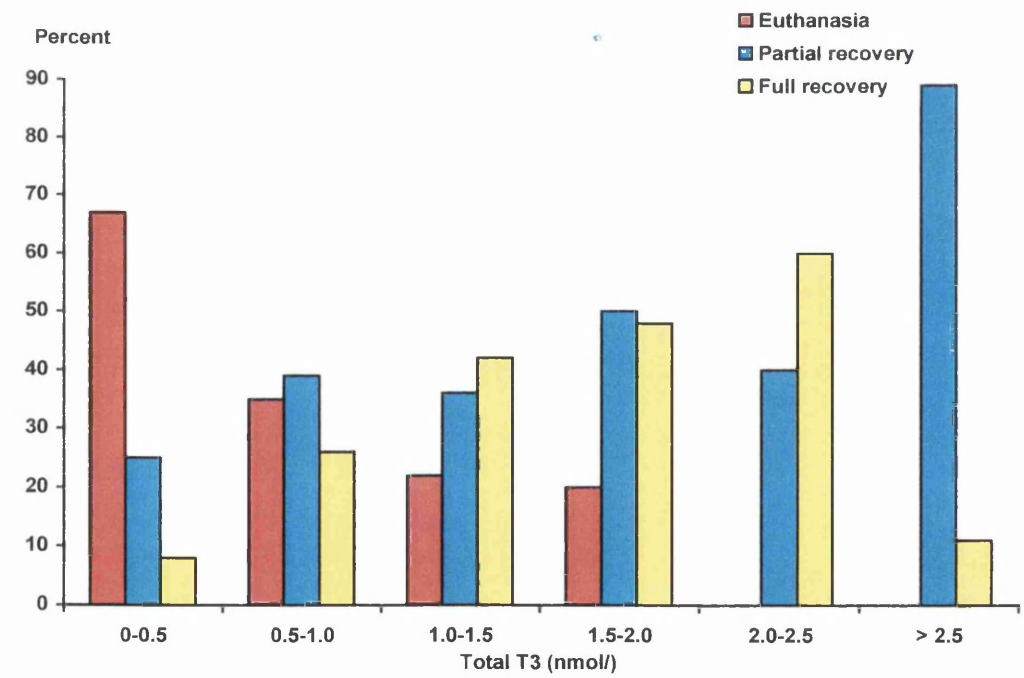


Figure 29. Circulating total T_3 concentrations in dogs with various diseases, categorised by eventual outcome. Results are grouped in 0.5 nmol/L bandings. The columns in each banding total 100 %.

Including the dogs which had received thyroid suppressive therapy, total T_4, free T_4 and total T_3 concentrations were decreased in 70 of 197 (35.5 %), eight of 179 (4.5 %) and 149 of 196 (76.0 %) cases, respectively. Total T_4 alone was reduced in three (1.5 %), total T_3 alone was reduced in 83 (42.3 %) and both hormones were concurrently reduced in 66 (33.7 %) of these dogs. Of the dogs which had not received thyroid suppressive therapies, total T_4, free T_4 and total T_3 were reduced in 39 of 131 (29.8 %), two of 120 (1.7 %) and 96 of 131 (73.3 %) cases, respectively. Total T_4 alone was reduced in two (1.5 %), total T_3 alone was reduced in 83 (45.0 %) and both hormones were concurrently reduced in 37 (28.2 %) of these dogs.

Since thyroid suppressive therapies were demonstrated to significantly alter total and free T_4 concentrations, analysis of the patterns of change of these parameters was performed after exclusion of those cases which had received these drugs. Circulating total T_4 concentrations were subnormal in 39 cases. These cases encompassed a wide range of disease types which are summarised in Table 39. Of these cases, 13 (33.3 %) were euthanatised, 16 (41.0 %) made a partial recovery and 10 (25.6 %) made a full recovery. Dogs with subnormal total T_4 values were significantly more likely to be euthanatised than dogs with normal total T_4 results. Free T_4 values were estimated in 36 of these 39 cases and were subnormal in only one (2.8 %) animal. This dog was ultimately euthanatised. Total T_3 was measured in 39 of the cases with subnormal total T_4, and was decreased in 37 (94.9 %) of them.

Two dogs had decreased circulating free T_4 concentrations and both of these cases were ultimately euthanatised. Circulating free T_4 values were at the low end of normal (less than 10 pmol/L) in nine dogs representing seven disease categories. Three of these cases were euthanatised, three made a partial recovery and three made a full recovery.

Of the 149 dogs with subnormal total T_3 values, one (0.7 %) died, 37 (24.8 %) were euthanatised, 57 (38.2 %) made a partial recovery and 54 (36.2 %) made a full recovery. Of these cases, 96 had not received thyroid suppressive therapy and total T_4 was decreased in 37 (38.5 %) of them whilst two (2.1 %) had concurrent subnormal free T_4 values. Only one dog with a normal total T_3 concentration was euthanatised. Dogs with subnormal total T_3 values were significantly more likely to be euthanatised than dogs with normal values.

Circulating cTSH concentrations were not significantly different between dogs with different outcomes. Circulating cTSH results were increased in six of 182 (3.3 %) cases which were suffering from orthopaedic, neoplastic or neurological diseases. The total T_4

concentration was concurrently decreased in two cases. Both of these cases had systemic neoplasia. All six dogs with elevated cTSH results had normal free T_4 values and were negative for TgAb.

Circulating TgAb were negative in 181 and equivocal in 18 dogs. No dogs were positive for TgAb. The distribution of TgAb results between case outcome groups is summarised in Table 40. The TgAb result was of no discriminatory value in assessing the likely outcome of cases.

Disease category	Number of dogs
Neoplasia	12
Gastrointestinal	7
Cardiovascular	3
Ophthalmological	3
Endocrine	2
General Medical	2
Neurological	2
Respiratory	2
Infectious	1
Immune mediated	1
Orthopaedics	1
Trauma	1
Urogenital	1
Unknown	1
Total	39

Table 39. Most common disease categories encountered amongst 39 dogs with subnormal total T_4 concentrations.

	TgAb Result		
	Positive	Equivocal	Negative
Died	0	0	1
Euthanased	0	2	36
Partial recovery	0	7	76
Full recovery	0	9	68

Table 40. Circulating TgAb results obtained from 199 dogs with various disorders categorised by eventual outcome.

8.4 DISCUSSION

The results of this study provided extensive data on the association of both clinical and endocrinological factors with the severity of illness and provide an insight into their use as prognostic indicators in dogs. To the author's knowledge, correlation of many of the features studied with eventual case outcome have not been previously reported in dogs.

The signalment of cases including age, gender and breed-type was similar to those of corresponding cases presented in Chapter 5. Approximately 19 % of all cases were ultimately euthanatised, compared to approximately 42 and 38 % which made partial or full recoveries, respectively. The low percentage of cases making a full recovery presumably reflects the type of cases referred for investigation. The author is unaware of corresponding outcome data for other referral institutions and these results provide data with which future studies can be compared.

It was clear that cases with neoplasia were more likely to be euthanatised than those with non-neoplastic disorders. Similarly less than 20 % of cases with neoplasia made complete recoveries. By contrast, orthopaedic cases were significantly more likely to make a full recovery. However, inadequate cases within the remaining disease categories hindered statistical evaluation and further studies evaluating a larger number of dogs are certainly warranted.

The duration of disease was of value in predicting the eventual case outcome and revealed that acute cases were significantly more likely to be euthanatised. However, as evidenced by the increased CDS in the acutely ill patients compared to those with chronic

conditions, those cases referred more rapidly after the development of signs were apparently suffering from more serious diseases and therefore this finding may be partly a consequence of dealing with a referral population. It is unclear if these results are applicable in first opinion practice where it is possible that more chronically affected cases may be less likely to recover. However, this issue is currently unclear and a similar evaluation of equivalent cases in a first opinion practice would be valuable and allow comparison with the results of the present study. As occurred during the analysis of the data presented in Chapter 5 and Appendix 31, the complicating effect introduced by analysis of purely referral data was acknowledged as a limitation of the present study. However, the results obtained in this study remain of value and provide novel data with which future studies can be compared.

The CDS was clearly a useful tool in predicting the eventual outcome of cases. The clinical value of this test was demonstrated by the sensitivity and specificity of 0.67 and 0.86, respectively for predicting euthanasia. Whilst clinical evaluation is normally a routine part of case assessment, it is usually performed in a fairly subjective manner. The assignment of an actual value for monitoring purposes may therefore assist in a more comprehensive and objective means of case evaluation.

It was clear from the results that younger dogs are more likely to make a full recovery than are older dogs. Whilst this may reflect an improved ability of the younger animal to cope during systemic illness, it is likely that these results are influenced by the nature of diseases which younger animals commonly suffer from, fewer of which are typically life-threatening. The results of the present study indicated that orthopaedics was more commonly associated with a full recovery than were the other disciplines. However, the data presented in Chapter 5 demonstrated that juvenile dogs are over-represented in certain referral disciplines including orthopaedics. This may explain the high recovery rates in young animals in the present chapter. As was advocated in the discussion in Chapter 5, a dedicated detailed study of population and disease demographics of referred dogs is a neglected area of research and is undoubtedly worthy of future study.

The effect of thyroid suppressive medication on circulating total and free T_4 concentrations was obvious in this study and supported the findings of previous studies (Woltz *et al.,* 1983; Torres *et al.,* 1991). Median total and free T_4 values were decreased by approximately 18 and 14 %, respectively compared to the values from dogs not receiving thyroid suppressive medications. However, there was no significant effect of these drugs on total T_3 or cTSH concentrations which is in contrast to the results of

previous reports (Rosychuk, 1982; Woltz *et al.*, 1983). The variation in some results of the present study with these reports may reflect variation in drug doses and duration of therapy, since it was not considered practical to evaluate these issues in detail in the present study. Clearly, of principal importance is the effect that commonly used drug regimes have on thyroid hormone parameters in a working clinical environment. Therefore, although a variety of drugs have been experimentally demonstrated to alter thyroid function, the results of this study indicate that these effects on total T_3 specifically may be of less importance in practice. The eventual outcome of cases in the present study was not significantly different between cases which had not been medicated, those which had received thyroid suppressive therapies, and those which had received other therapies prior to referral. Therefore, the demonstration of a strong correlation between total and free T_4 concentrations and the likelihood of recovery was not due to the complicating effect of previous therapies on thyroid hormone concentrations.

The most common pattern of thyroid abnormalities encountered was a reduction in total T_3 alone which occurred in over 40 % of patients studied. Concurrent reduction of both total T_4 and total T_3 was also common occurring in approximately one third of dogs. However the prevalence of subnormal total T_4 alone was very low. These results are interesting and are broadly similar to the changes which occur in the low T_3 syndrome in humans with NTI (Bermudez *et al.*, 1975; Nicoloff & LoPresti, 1996). The reduced frequency of subnormal total T_4 compared to total T_3 values may indicate that the T_4 depression associated with systemic illness in dogs occurs later in the course of disease. In support of this was the finding that the frequency of subnormal total T_3 in cases with depressed total T_4 values was nearly 100 % whereas only approximately 40 % of dogs with subnormal total T_3 values had a concurrent decrease in total T_4 concentration. However, this remains unclear and longitudinal case studies evaluating the change in hormone concentrations with disease progression would be of considerable value in this regard. These results are interesting since most previous studies in dogs have reported that total T_3 is less consistently decreased in NTI than is total T_4 (Peterson *et al.*, 1984; Larsson, 1988; Elliot *et al.*, 1995; Peterson *et al.*, 1997). There are several possible explanations for the discrepancy of these reports with the present study. Many of the previous reports of canine thyroid function in NTI have evaluated specific types of illness, usually either diseases mimicking hypothyroidism (Larsson, 1988; Peterson, 1997) or frequently, dogs with hyperadrenocorticism (Peterson *et al.*, 1984). Unlike the present study or contemporary human reports, it has been rare for canine studies to evaluate wide ranging disease

categories. Differences in the cases evaluated may therefore partly account for the difference in results. This is certainly supported by the data presented in Chapter 4, in which less than 20 % of cases with NTI which clinically mimicked hypothyroidism, had subnormal total T_3 concentrations. In particular, the reliance on dogs with hyperadrenocorticism which is largely mediated by glucocorticoid excess is unlikely to truly represent the pattern of thyroid hormone abnormalities in general NTI. This is supported by the increased prevalence of subnormal total T_3 values reported in this disease (Peterson *et al.,* 1984) compared with dogs with a wider range of illnesses (Larsson, 1998; Peterson *et al.,* 1997). The frequent reduction in total T_3 values in hyperadrenocorticism are presumably a consequence of the reduced peripheral conversion of T_4 to T_3 associated with glucocorticoid excess (Gambert, 1996). As outlined previously, the high prevalence of subnormal total T_4 concentrations in hyperadrenocorticism is likely related to a combination of abnormalities including suppression of thyrotropin secretion and altered affinity and concentration of hormone binding proteins. The frequent use of hyperadrenocorticism as a model of metabolic disease must certainly be in question. The report by Elliot *et al.,* (1995) which evaluated a wide range of diseases was more comparable to the present study and correspondingly the reported frequency of abnormalities of total T_4 and particularly total T_3 values were closer to those found in the present study. An alternative explanation includes the reference ranges used to identify "normal" from "abnormal" values in different studies. Commonly, previous studies have compared hormone results with those obtained from a putatively healthy group of dogs (Larsson, 1988; Peterson *et al.,* 1997). However, in the present study, the cut-off value used to classify total T_3 results as abnormal was the concentration which most reliably distinguished between hypothyroidism and NTI identified in Chapter 4. Whilst both approaches are justifiable, the advantage of the limits used in the present study, are that the cut-off used was generated using a "control" population of dogs which all had various NTI. Therefore, the identification of a subnormal value in the dogs with NTI in the present chapter, is genuinely separating those dogs based on the severity of their illness, rather than simply the presence or absence of illness as occurs in most other studies. This was considered to be a more appropriate approach to this problem, and typifies the complexities involved in selecting an appropriate control group as highlighted in Chapter 5.

The correlation of thyroid hormone concentrations with eventual outcome demonstrated that both total and free T_4 and total T_3 concentrations decrease in NTI and the magnitude of depression is strongly associated with the likelihood of euthanasia. This is

similar to reports in humans and cats (Slag *et al.*, 1981; Mooney *et al.*, 1996). Of all the parameters of thyroid function evaluated, total T_3 concentrations were most strongly correlated with eventual outcome. In the dogs that were euthanatised the mean value was reduced to only approximately 60 % of that in the dogs which survived. Identification of a normal total T_3 value was highly predictive of survival although like all the parameters studied, this could not differentiate between the likelihood of a partial or full recovery. However, identification of a subnormal total T_3 result was associated with a mortality rate which was similar to that of all dogs in the study. Therefore, at the cut-off value selected in this study, total T_3 estimation can apparently be used as a specific predictor of recovery.

The effect of NTI on total T_4 values was also marked, with values being reduced in the patients that were subsequently euthanatised to approximately half that of the dogs which made a full recovery. Total T_4 values were subnormal in 29.8 % of cases which had not received any thyroid suppressive therapy compared to 35.5 % of all cases including those which had received these drugs. Euthanasia was significantly more likely in dogs with a subnormal total T_4 concentration compared to dogs with normal values. This is similar to reports in other species (Slag *et al.*,1981; Mooney *et al.*, 1996) but is in contrast to the report by Elliot *et al.*, (1995) in which no correlation of total T_4 and outcome was identified. The reason for the discrepancy of the present study with that of Elliot *et al.*,(1995) probably reflects an increased incidence of subnormal total T_4 values in the surviving dogs reported by Elliot *et al.*, (1995) which may have been present due to the type of cases studied as previously discussed. The dogs with depressed total T_4 concentrations in the present study encompassed a wide range of disease categories indicating that the use of thyroid hormone estimation as a prognostic marker is of value in a wide range of clinical illnesses.

Although also significantly reduced in non-surviving compared with surviving patients, the free T_4 concentration was less markedly affected by NTI decreasing by approximately one third in those dogs which were euthanatised compared with dogs which made a full recovery This is similar to the results of human studies but has not been previously reported in dogs with wide ranging illnesses. Analysis of the likelihood of euthanasia in dogs with subnormal free T_4 values could not be evaluated due to an inadequate number of cases. Subnormal free T_4 values occurred in only 1.7 % of cases which had not received thyroid suppressive therapy supporting the conclusion in Chapter 4 that identification of a subnormal free T_4 is a specific indicator of thyroid disease. Because only two cases had subnormal free T_4 values interpretation of these results must be

performed cautiously. However, both cases were ultimately euthanatised suggesting that although uncommon, a subnormal free T_4 concentration may be a very poor prognostic indicator in NTI. Further studies with additional cases are clearly required.

Measurement of circulating TgAb was of no prognostic utility in the cases studied. The absence of a positive TgAb result in any of the samples collected supports the conclusions in Chapter 7, that measurement of this analyte is a highly specific method for the identification of thyroid disease.

In conclusion, this study has demonstrated that the most common pattern of thyroid hormone changes in dogs with a wide range of NTI is depression of total T_3 alone. Reduction of both total T_4 and total T_3 concentrations is also common. It is clear that the severity of illness in dogs as assessed by the likelihood of recovery, can be inferred from estimation of thyroid hormone concentrations. Specifically, the maintenance of total T_3 concentrations in the face of illness, is a highly specific indicator that recovery is likely. However a reduction in total T_3 is not uncommon in cases which ultimately survive. Circulating total T_4 concentrations decrease in approximately 30 % of cases irrespective of the type of illness and more marked depressions in this hormone are associated with reduced likelihood of recovery. In contrast, although free T_4 is also usually decreased by illness, the reduction is less than for total T_4 and subnormal values are only rarely encountered. However, it is possible that a subnormal free T_4 concentration is a very poor prognostic indicator. The clinical impression of the severity of illness correlates well with eventual outcome as does the age of dog and duration of illness, with older and more acutely ill patients having a markedly poorer prognosis. Not surprisingly, cases with neoplasia are much more likely to die as a result of their illness than cases with non-neoplastic diseases.

CHAPTER 9

GENERAL CONCLUSIONS AND FUTURE RESEARCH

9.1. INTRODUCTION

The objectives of this study were outlined in Chapter 1 and the relevant conclusions have been detailed in the appropriate chapters. This chapter serves to summarise the most significant findings and highlight the areas which have been recognised as areas worthy of further research.

9.2 ASSAY VALIDATION

Assays for total and free T_4, total T_3, cTSH and TgAb were successfully validated for use with canine serum. In the case of the total T_4 assay, this was achieved despite modification to allow determination of the lower concentrations typically encountered in dogs compared with humans. The confirmation that the cTSH assay is a valid canine method is a major advancement in the study of canine thyroid disease. The ability to accurately determine cTSH has been awaited for several decades, and the importance of the release of this assay has been highlighted by the large number of publications in this area since the present study commenced. However, it was recognised that the current cTSH assay remains too insensitive to allow the identification of subnormal cTSH values therefore reducing its utility in the confirmation of central hypothyroidism or in accurate therapeutic monitoring. As occurred with the equivalent human TSH assays more than 20 years ago, further commercial development of this assay is anticipated in the future and appropriate studies in these areas will be required at that time. The confirmation of a valid TgAb assay is arguably the most significant step forward in the study of canine thyroid disease since the development of methods for the estimation of the thyroid hormones themselves. The ability to identify developing thyroid disease in its earliest stages, potentially prior to the occurrence of clinical abnormalities, has profound implications for the management of both healthy dogs and those with suspected hypothyroidism in veterinary practice in the coming years. The opportunity to make informed breeding decisions in healthy dogs based on TgAb results is one example of the role that this test is likely to assume in the future. However, many questions in this area still exist. The association, if any, between autoimmune thyroiditis and idiopathic atrophy needs to be clarified and it is likely that TgAb estimation will greatly facilitate this. The time taken for disease progression in

individuals is currently unclear and also requires longitudinal case studies and TgAb monitoring programmes will be invaluable. Study of the underlying genetic predisposition to canine hypothyroidism will also be assisted by monitoring TgAb within particular families of dogs.

9.3 DIAGNOSIS OF CANINE HYPOTHYROIDISM

This study has reported the diagnostic utility of total T_4, free T_4, total T_3 and cTSH for the confirmation of canine hypothyroidism. The use of DPR analysis and ROC curve generation have also been shown to be dynamic statistical methods which can be practically applied to this type of study. It is clear that as previously reported, total T_4 and total T_3 are poorly specific indicators of thyroid status and are frequently affected by NTI and drug therapy. In contrast, the estimation of free T_4 has been shown to be the single most accurate test and a more specific indicator of thyroid function. The role of cTSH estimation, not as a solitary diagnostic test, but as a supporting test has also been proven to significantly enhance the ability of the clinician to reliably confirm hypothyroidism. Features which in practice are prone to interfering with these tests have been identified and include recovery from NTI and the use of sulphonamide-containing drug therapies. Consequently, diagnostic guidelines to facilitate the accurate diagnosis of hypothyroidism have now been established which can be widely applied in practice. Whilst many questions regarding the diagnosis of hypothyroidism were answered in this study, just as many became apparent during the course of the work. The reason for normal cTSH concentrations in a substantial number of dogs with hypothyroidism remains unclear and requires further study. Various explanations have been summarised previously but research into both technical causes related to the assay itself and biological causes will be undoubtedly be rich sources of future studies.

9.4. EPIDEMIOLOGY AND ROUTINE CLINICAL PATHOLOGY

For the first time in the UK the epidemiological characteristics of hypothyroidism, confirmed using a reliable diagnostic method, have been identified. The breeds and ages of affected dogs were similar to those identified in other parts of the world including the USA and Australia. However, limitations of the present study due to the nature of the referral population studied were acknowledged, and future studies to minimise the effect of such errors are required. However, this was a conclusion which applied not simply to the identification of breed or pedigree predilections for hypothyroidism since the complexities

associated with identification of suitable control populations were also apparent in the estimation of endocrine parameters and routine biochemical and haematological analytes. It became evident that even some of the most basic information on the demographics of the UK canine population is currently unclear, hindering epidemiological analysis of canine disease in general. It is hoped that this area will be comprehensively evaluated in the near future, since it has implications for many areas of clinical study in dogs. The routine clinicopathological features associated with hypothyroidism were identified and in some cases confirmed previous reports. However, the use of newer biochemical tests such as fructosamine in dogs with hypothyroidism revealed the potential for improved performance of routine tests in this condition. The limitations of routine biochemistry and haematology evaluation were also clearly demonstrated by use of a novel approach based on measurement of these parameters in dogs with clinically similar NTI. Consequently, guidelines on routine diagnostic testing were produced which will be of considerable value in routine practice.

9.5. THERAPEUTIC MONITORING

Previous reports concerning therapy and therapeutic monitoring for hypothyroidism have produced conflicting guidelines and consequently this area has previously been a source of debate. The results of this study demonstrated that once daily THRT using a synthetic T_4 preparation is a successful method for resolution of clinical and clinicopathological abnormalities. The need for twice daily therapy has been shown to be rare thus the guidelines produced improve the likelihood of client compliance and facilitate a more economic approach to therapy. The use of six hour post-pill peak total T_4 concentrations as a monitoring tool has been confirmed, but unlike the variation in guidelines for therapeutic monitoring which has previously been a common difficulty, objective hormone concentrations which are correlated with clinical outcome have been identified and reported. Circulating cTSH estimation during therapeutic monitoring has been shown to be of limited value but as identified previously, this is an area which may assume greater importance in the future as commercial assays are progressively modified. Novel data on the biochemical and haematological changes which occur in dogs receiving THRT have been reported for the first time. This study has therefore provided a basis from which future studies can develop and be compared. The potential for use of routine clinicopathological parameters such as creatinine in therapeutic monitoring has been investigated and based on the results clearly warrants further evaluation. It is quite possible that this section of the

study alone has therefore identified an area which may ultimately become of substantial economic and practical benefit to veterinary practitioners and dog owners alike.

9.6. THYROGLOBULIN AUTOANTIBODIES

The reliable determination of circulating TgAb in healthy dogs, those with hypothyroidism and those with clinically similar NTI has been evaluated in detail for the first time. The results confirm the high specificity of a positive TgAb result for thyroid disease, and as discussed previously have revealed that research in this area is necessary and justified.

9.7. THYROID FUNCTION IN NON-THYROIDAL ILLNESS

The role of thyroid hormone parameters in NTI has been studied in detail in humans and briefly in cats. However, previously only one limited study had evaluated the role of tests of thyroid function in dogs with NTI. The results of the present study have, for the first time, clearly demonstrated the marked association between total and free thyroid hormone concentrations and the likelihood of recovery in a wide range of diseases. The use of thyroid hormones as prognostic markers in dogs is therefore clearly an area which may have profound implications for canine welfare and is undoubtedly worthy of future study. In addition, the identification of clinical parameters which are of prognostic relevance has also been reported. Whilst this was almost an incidental finding in the principal study, it revealed an area which is largely unstudied and based on the findings requires further and more detailed consideration. The pattern of thyroid hormone abnormalities encountered in NTI has also been identified and in some respects differs from previously held assumptions. The limitations of previous studies have been highlighted and it is expected that the publication of the results of the present study will stimulate considerable interest and future research in this area over the coming years.

LIST OF APPENDICES

Appendix 1

Registration information recorded to the hospital computer system (Dataflex).

Field	Comments
Hospital Number	
Animal Name	
Species	
Breed 1	e.g. terrier, collie etc.
Breed 2	e.g. west highland white, rough etc.
Date of Birth	
Age	Updated by hospital system
Sex	Including neutering status
Date First Seen	
Date Last Seen	
Visit Number	
Owner Reference	Unique to owner
Key	Owner name and initials
Name	Owner name and title
Address	Owner address
Town	
Code	Owner address postal code
Home Tel	Owner home telephone number
Bus Tel	Owner business telephone number
Fax	Owner home facsimile number
Email	Owner electronic mail address
Vet Ref	Unique numerical reference of referring veterinary surgeon
Key	Referring veterinary surgeon's name and initials
Name	Referring veterinary surgeon's name
Address	Referring veterinary surgeon's address
Town	
Code	Referring veterinary surgeon's postal code
Tel	Referring veterinary surgeon's telephone number
Fax	Referring veterinary surgeon's facsimile number
Email	Referring veterinary surgeon's electronic mail address
Clin Ref	Unique numerical reference of clinician
Key	Clinician's unique text code e.g. RMD, CTM etc.
Name	Clinician's name
Dept	Division within Department of Veterinary Clinical Studies

Appendix 2

Measured and calculated routine haematology parameters.

Parameter	Units	Comments
RBC	x 10^{12}/L	Red blood cell count
Hb	g/dl	Haemoglobin
HCT	%	Haematocrit
MCV	fl	Mean red cell volume
MCH	pg	Mean red cell haemoglobin
MCHC	g/dl	Mean red cell haemoglobin concentration
PLT	x 10^9/L	Platelet count
MPV	fl	Mean platelet volume
PCT	%	Plateletcrit
PDW		Platelet distribution width measured in arbitrary units at 20% of the maximum height of the platelet histogram
WBC	x 10^9/L	White blood cell count
Band Neutrophils	x 10^9/L	
Neutrophils	x 10^9/L	
Lymphocytes	x 10^9/L	
Monocytes	x 10^9/L	
Eosinophils	x 10^9/L	
Basophils	x 10^9/L	

Appendix 3

Measured routine biochemical parameters.

Parameter	Units	Analyser	Methodology
Alanine Aminotransferase	IU/L	Axon	L-alanine and α-ketoglutarate coupled to lactate dehydrogenase activity
Albumin	g/L	Axon	Bromocresol green dye binding with rapid absorbance
Alkaline Phosphatase	IU/L	Axon	p-nitrophenol phosphate in DEA buffer
Aspartate Aminotransferase	IU/L	Axon	L-aspartate and α-ketoglutarate coupled to malate dehydrogenase activity
Bilirubin	μmol/L	Axon	Diazotised sulphanilic acid
Calcium	mmol/L	Axon	Cresolphthalein complexone
Chloride	mmol/L	Axon	Ferric thiocyanate
Cholesterol	mmol/L	Axon	Cholesterol esterase, cholesterol oxidase and peroxidase
Creatine Kinase	IU/L	Mira	N-acetylcysteine, hexokinase and glucose-6-phosphate dehydrogenase
Creatinine	μmol/L	Axon	Picric acid
Fructosamine	μmol/L	Mira	Nitroblue tetrazolium
γ-Glutamyl Transferase	IU/L	Axon	γ-Glutamyl-p-nitroanilide and glycylglycine
Globulin	g/L	Axon	Arithmetic subtraction (total protein minus albumin)
Glucose	mmol/L	Axon	Glucose hexokinase and D-glucose-6-phosphate dehydrogenase
Inorganic Phosphorus	mmol/L	Axon	Ammonium molybdate
Magnesium	mmol/L	Axon	Xylidyl blue
Potassium	mmol/L	Axon	Ion selective electrode
Sodium	mmol/L	Axon	Ion selective electrode
Total Protein	g/L	Axon	Biuret reagent
Triglycerides	mmol/L	Axon	Lipoprotein lipase, glycerol kinase, glycerol phosphate oxidase and peroxidase
Urea	mmol/L	Axon	Urease and glutamic dehydrogenase

Axon Technicon Axon (Bayer)
Mira Cobas Mira (Roche)

Appendix 4

Data categories used to store additional case information at each dog-visit

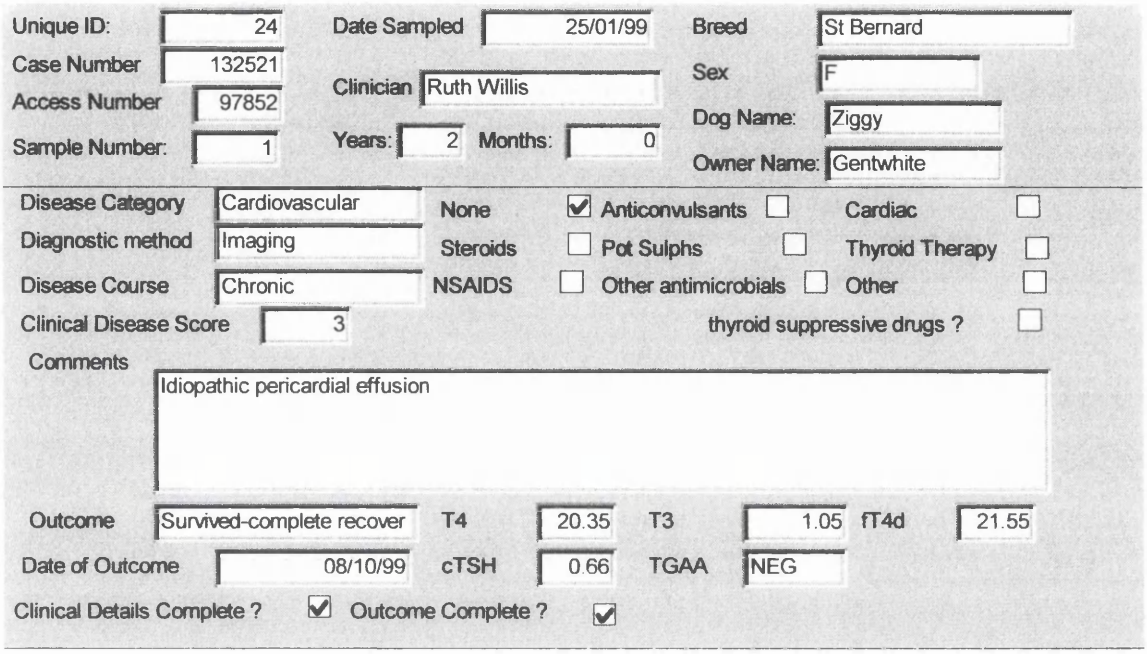
Category	Comments
Hospital Number	Unique six figure hospital number
Stim	Clinical biochemistry laboratory identification relating to bovine thyrotropin stimulation test (if performed on that visit)
Stim Date	Date of thyrotropin stimulation test (if performed on that visit)
Routine	Clinical biochemistry laboratory identification relating to routine profile (if performed on that visit)
Routine Date	Date of routine profile (if performed on that visit)
Name	Owner name
Age	Animal age
Sex	Sex and neutering status
Breed	Breed
Weight	Weight
HypoT_4	Numerical coding (1-hypothyroid, 0-euthyroid)
Other Diagnosis	Other relevant clinical information
Visit No	Visit number since first visit
Wks on Tx	Number of weeks on L-thyroxine therapy (if appropriate)
Basal T_4	Basal or pre-thyrotropin total thyroxine concentration
Post TSH T_4	Post-thyrotropin total thyroxine concentration
cTSH	Endogenous thyrotropin concentration
6 hr post pill	Six hour post-L-thyroxine total thyroxine concentration (hypothyroid dogs on treatment only)
fT_4d	Free thyroxine concentration
fT_4d Date	Date of free thyroxine assay
Box	Serum sample storage location identification
Drug Tx	Current or recent drug therapy
Urea	Routine profile result (urea)
Na	Routine profile result (sodium)
K	Routine profile result (potassium)
Cl	Routine profile result (chloride)
Ca	Routine profile result (calcium)
Mg	Routine profile result (magnesium)
PO_4	Routine profile result (inorganic phosphate)
Gluc	Routine profile result (glucose)
Chol	Routine profile result (cholesterol)
Crea	Routine profile result (creatinine)
Tbil	Routine profile result (total bilirubin)
ALKP	Routine profile result (alkaline phosphatase)
AST	Routine profile result (aspartate aminotransferase)
ALT	Routine profile result (alanine aminotransferase)
TP	Routine profile result (total protein)
Alb	Routine profile result (albumin)
Glob	Routine profile result (globulin)

Appendix 4 (continued)

Category	Comments
A:G	Routine profile result (albumin to globulin ratio)
CK	Routine profile result (creatine kinase)
Trig	Routine profile result (triglycerides)
GGT	Routine profile result (γ-glutamyl transferase)
Fruct	Routine profile result (fructosamine)
RBC	Routine profile result (red blood cell count)
Hb	Routine profile result (haemoglobin)
HCT	Routine profile result (haematocrit)
MCV	Routine profile result (mean red blood cell volume)
MCH	Routine profile result (mean red blood cell haemoglobin)
MCHC	Routine profile result (mean red blood cell haemoglobin concentration)
PLT	Routine profile result (platelet count)
MPV	Routine profile result (mean platelet volume)
WBC	Routine profile result (white blood cell count)
Band N	Routine profile result (band neutrophils)
Neut	Routine profile result (neutrophils)
Lymp	Routine profile result (lymphocytes)
Mono	Routine profile result (monocytes)
Eosin	Routine profile result (eosinophils)
Baso	Routine profile result (basophils)
Misc	Miscellaneous comments field

Appendix 5

Example of completed template used to record historical and clinical data from hospitalised cases with various disorders.



Appendix 6

Construction of the Receiver Operating Characteristic (ROC) curve and calculation of the area under the ROC curve (W)

The calculation of the area under the ROC curve (W) and the calculation of the standard error of W as exemplified by cut-off values of T_4 for the confirmation of hypothyroidism.

	Cut-Off Value (nmol/L)								
	7.6	**8.2**	**8.3**	**8.5**	**9.1**	**9.6**	**9.8**	**10.1**	**10.2**
Nx	0	1	2	1	0	0	1	2	1
Nax	85	84	82	81	81	81	80	78	77
Ax	1	0	0	0	1	1	0	0	0
Abx	36	37	37	37	37	38	39	39	39
$W_{partial}$	0	37	74	37	0	0	39	78	39
$Q1_{partial}$	0	1369	2738	1369	0	0	1521	3042	1521
$Q2_{partial}$	7225	0	0	0	6561	6561	0	0	0

Nx Number of dogs without hypothyroidism having a test result at the cut-off value

Nax Number of dogs without hypothyroidism having a test result above the cut-off value

Ax Number of dogs with hypothyroidism having a test result at the cut-off value

Abx Number of dogs with hypothyroidism having a test result below the cut-off value

At each cut-off value the partial terms of the area under the ROC curve ($W_{partial}$) and of the intermediate probabilities $Q1_{partial}$ and $Q2_{partial}$ are calculated using the formulae:

$$W_{partial} = (Nx * Abx) + (½ * Nx * Ax)$$

$$Q1_{partial} = Nx * [(Abx * Abx) + (Abx * Ax) + (Ax * Ax / 3)]$$

$$Q2_{partial} = Ax * [(Nax * Nax) + (Nax * Nx) + (Nx * Nx / 3)]$$

When $W_{partial}$, $Q1_{partial}$, and $Q2_{partial}$ have been calculated for each cut-off value, the final value of W and of the intermediate probabilities Q1 and Q2 are calculated using the

following formulae in which Nd represents the number of dogs with hypothyroidism and Nn represents the number of dogs without hypothyroidism.

$W = (\Sigma W_{partial}) / (Nd * Nn)$

$Q1 = (\Sigma Q1_{partial}) / (Nd * Nd * Nn)$

$Q2 = (\Sigma Q2_{partial}) / (Nn * Nn * Nd)$

The standard error of the area under the ROC curve (SEw) is then calculated as:

$$\sqrt{\frac{(W * [1-W]) + ([Nd-1] * [Q1-W^2]) + ([Nn-1] * [Q2-W^2])}{Nd * Nn}}$$

Appendix 7

Total thyroxine (T_4) intra-assay precision.

	Total T_4 Concentration (nmol/L)		
Duplicate	**Low**	**Intermediate**	**High**
1	15.1	37.2	95.1
2	16.7	31.7	91.1
3	16.2	34.7	92.9
4	16.7	35.1	98.4
5	15.5	32.5	92.3
6	16.6	36.5	90.7
7	16.8	40.2	91.7
8	15.0	37.4	87.9
9	15.6	36.6	93.7
10	15.7	42.6	96.0
Mean	16.0	36.5	93.0
s.d.	0.7	3.3	3.0
c.v. (%)	4.3	9.0	3.2

Appendix 8

Total thyroxine (T_4) inter-assay precision.

	Total T_4 Concentration (nmol/L)		
Assay Run	**Low**	**Intermediate**	**High**
1	5.5	14.0	172.0
2	5.1	13.8	170.8
3	4.3	11.4	155.9
4	5.7	11.5	169.9
5	6.2	15.8	168.0
6	4.5	13.3	156.0
7	4.8	13.6	168.8
8	6.7	16.9	179.4
9	5.1	10.3	158.9
10	6.3	13.5	164.6
Mean	5.4	13.4	166.4
s.d.	0.8	2.0	7.6
c.v. (%)	14.8	14.9	4.6

Appendix 9

Total thyroxine (T_4) linearity study using the supplied zero standard as the diluent.

	Total T_4 concentration (nmol/L)		
Progressive 20% Dilutions	**Observed (O)**	**Expected (E)**	**% O/E**
Undiluted	21.2		
Dilution 1	17.9	17.0	105.5
Dilution 2	16.0	14.3	111.7
Dilution 3	13.4	12.8	104.7

Appendix 10

Total thyroxine (T_4) limit of detection.

Replicate	CPM
1	50350
2	50259
3	50000
4	50257
5	50508
6	49739
7	50559
8	50124
9	50154
10	50600
11	50935
12	50455
13	50536
14	50320
15	50350
16	50320
17	50210
18	50358
19	51232
20	50241
Mean	50375
s.d.	318
Mean - 2.6 s.d.	49549
Equivalent total T_4 limit of detection	4.7 nmol/L

CPM: Counts Per Minute

Appendix 11

Free thyroxine measured by dialysis intra-assay precision.

	Free T_4 Concentration (pmol/L)		
Duplicate	**Low**	**Intermediate**	**High**
1	9.4	25.3	42.7
2	9.8	20.9	45.9
3	9.7	20.2	45.6
4	8.7	17.7	44.4
5	11.9	20.3	46.1
6	11.5	20.1	44.5
7	10.2	20.3	45.9
8	9.2	20.4	50.2
9	9.6	18.6	45.5
10	14.1	18.2	48.4
Mean	10.4	20.2	45.9
s.d.	1.6	2.1	2.1
c.v. (%)	15.7	10.3	4.6

Appendix 12

Free thyroxine measured by dialysis inter-assay precision.

	Free T_4 Concentration (pmol/L)		
Assay Run	**Low**	**Intermediate**	**High**
1	11.3	22.6	164.6
2	13.4	25.6	223.2
3	11.2	22.3	179.9
4	10.3	24.7	197.1
5	13.0	25.2	185.3
6	14.7	26.3	201.8
Mean	12.3	24.4	192
s.d.	1.7	1.6	20.2
c.v. (%)	13.5	6.7	10.5

Appendix 13

Free thyroxine by dialysis linearity study using the supplied zero standard as the diluent.

	Free T_4 concentration (pmol/L)		
Dialysate Dilution	**Observed (O)**	**Expected (E)**	**% O/E**
Undiluted	126.6		
1:1	58.1	63.3	91.8
1:3	29.4	29.0	101.2
1:7	15.9	14.7	108.2

Appendix 14

Free thyroxine measured by dialysis limit of detection.

Replicate	CPM
1	11549
2	11452
3	11965
4	11260
5	11898
6	11126
7	11728
8	10861
9	12358
10	12292
11	12259
12	11197
13	12356
14	11677
15	12409
16	11804
17	12477
18	11798
19	12009
20	11399
Mean	11793
s.d.	477
Mean + 2.6 s.d.	13036
Equivalent free T_4 limit of detection	3.22 pmol/L

CPM: Counts Per Minute

Appendix 15

Total triiodothyronine (T_3) intra-assay precision.

	Total T_3 Concentration (nmol/L)		
Duplicate	**Low**	**Intermediate**	**High**
1	1.24	2.40	4.38
2	1.21	2.23	4.45
3	1.42	2.35	4.33
4	1.34	2.39	4.54
5	1.15	2.17	4.46
6	1.27	2.28	4.48
7	1.26	2.30	4.42
8	1.28	2.38	4.50
9	1.25	2.50	4.47
10	1.30	2.27	3.74
Mean	1.27	2.33	4.38
s.d.	0.07	0.10	0.23
c.v. (%)	5.7	4.1	5.3

Appendix 16

Total triiodothyronine (T_3) inter-assay precision.

	Total T_3 Concentration (nmol/L)		
Assay Run	**Low**	**Intermediate**	**High**
1	1.59	2.23	3.80
2	1.46	1.92	4.00
3	1.77	2.19	4.14
4	1.59	1.91	not done
Mean	1.60	2.06	3.98
s.d.	0.13	0.17	0.17
c.v. (%)	7.9	8.3	4.3

Appendix 17

Total triiodothyronine (T_3) linearity study using the supplied zero standard as the diluent.

	Total T_3 concentration (nmol/L)		
Dilution	**Observed (O)**	**Expected (E)**	**% O/E**
Undiluted	4.40		
1:1	2.08	2.20	94.6
1:3	0.98	1.04	94.2
1:7	0.55	0.49	112.2

Appendix 18

Total triiodothyronine (T_3) limit of detection.

Replicate	CPM
1	18158
2	18412
3	17880
4	18479
5	18259
6	18922
7	18296
8	18177
9	18233
10	17865
11	18126
12	18127
13	18315
14	18154
15	18088
16	18430
17	17623
18	18244
19	17732
20	18161
Mean	18184
s.d.	282.5
Mean - 2.6 s.d.	17450
Equivalent total T_3 limit of detection	0.14 nmol/L

CPM: Counts Per Minute

Appendix 19

Thyrotropin (cTSH) intra-assay precision.

	cTSH Concentration (ng/ml)		
Duplicate	**Low**	**Intermediate**	**High**
1	0.12	0.84	2.13
2	0.14	0.86	2.06
3	0.13	0.87	217
4	0.14	0.82	2.21
5	0.13	0.83	2.22
6	0.14	0.89	2.21
7	0.16	0.84	2.20
8	0.13	0.86	2.13
9	0.14	0.83	2.16
10	0.14	0.84	2.18
Mean	0.137	0.848	2.167
s.d.	0.011	0.021	0.049
c.v. (%)	7.7	2.5	2.3

Appendix 20

Thyrotropin (cTSH) inter-assay precision.

	cTSH Concentration (ng/ml)		
Assay Run	**Low**	**Intermediate**	**High**
1	0.14	0.44	1.34
2	0.15	0.42	1.40
3	0.16	0.46	1.27
4	0.12	0.43	1.28
5	0.16	0.38	1.18
6	0.15	0.43	1.24
7	0.15	0.39	1.19
8	0.08	0.37	1.34
9	0.10	0.37	1.22
10	0.11	0.28	1.24
Mean	0.132	0.396	1.27
s.d.	0.029	0.052	0.07
c.v. (%)	21.9	13.2	5.6

Appendix 21

Thyrotropin (cTSH) linearity study using the supplied zero standard as the diluent.

Dilution	Observed (O) (ng/ml)	Expected (E) (ng/ml)	% O/E
Undiluted	2.84		
1:1	1.26	1.42	88.7
1:3	0.68	0.63	107.9
1:7	0.30	0.34	88.2

Appendix 22

Thyrotropin (cTSH) limit of detection.

Replicate	CPM
1	51
2	60
3	49
4	56
5	42
6	52
7	42
8	39
9	60
10	52
11	51
12	42
13	46
14	41
15	52
16	58
17	42
18	44
19	44
20	43
Mean	48.3
s.d.	6.7
Mean + 2.6 s.d.	65.7
Equivalent cTSH limit of detection	0.01 ng/ml

CPM: Counts Per Minute

Appendix 23

Quantitative thyroglobulin autoantibody (TgAb) intra-assay precision results. Figures shown are optical density values expressed as a percentage of the negative control sample optical density.

	Sample 1 (positive)	**Sample 2 (negative)**
	967	108
	938	94
	904	99
	896	97
	907	98
	892	90
	876	95
	908	82
	1075	91
	1001	82
	1000	105
	999	87
	1006	117
	941	105
	1045	98
	1055	84
	958	91
	1007	94
	1048	99
	1084	104
Mean	975	96
s.d.	65.9	9.06
c.v. (%)	6.75	9.44

Appendix 24

Quantitative thyroglobulin autoantibody (TgAb) inter-assay precision results. Figures shown are optical density values expressed as a percentage of the negative control sample optical density.

Assay Run	Positive	Equivocal	Negative
1	1105	136	58
2	1139	200	100
3	1491	161	76
4	983	192	58
5	1080	130	59
6	975	96	42
Mean	1129	152	66
s.d.	189	39.6	20.1
c.v. (%)	16.8	26.0	30.7

Appendix 25

Qualitative thyroglobulin autoantibody (TgAb) intra-assay precision.

Duplicate	Sample 1	Sample 2
1	Negative	Positive
2	Negative	Positive
3	Negative	Positive
4	Negative	Positive
5	Negative	Positive
6	Negative	Positive
7	Negative	Positive
8	Negative	Positive
9.	Negative	Positive
10	Negative	Positive
Qualitative Precision	100 %	100 %

Appendix 26

Qualitative thyroglobulin autoantibody (TgAb) inter-assay precision.

Assay Run	Sample 1	Sample 2	Sample 3
1	Negative	Equivocal	Positive
2	Negative	Equivocal	Positive
3	Negative	Equivocal	Positive
4	Negative	Equivocal	Positive
5	Negative	Equivocal	Positive
6	Negative	Negative	Positive
Qualitative Precision	100 %	83 %	100 %

Appendix 27

Classification of cases with equivocal TSH response test results

Three hypothyroid and nine euthyroid dogs exhibited an equivocal response to bovine TSH administration. The initial hormone results from these cases are detailed in Tables 41 and 42, respectively.

In one of the three dogs ultimately confirmed as hypothyroid (130239) there was a further decline in total T_4 (from 13.8 to 3.0 nmol/L) and increase in cTSH concentration (from 0.94 to 1.32 ng/ml) over a period of 14 weeks. In another of these cases (122647) there was evidence of lymphocytic thyroiditis on histopathological examination of thyroid tissue. In the third (129912), the owner circumstances were such that T_4 replacement therapy was the only available method for supporting or refuting hypothyroidism. In this and the other two cases there was an unequivocal response to T_4 supplementation without the need for other therapy.

Of the nine euthyroid dogs with equivocal TSH response test results, there was a complete resolution from non-thyroidal illness following appropriate therapy in eight cases (121319, 125089, 127515, 127978, 129496, 132278, 132320 and 132467). In the remaining case (129960) there was a return of total T_4 and cTSH to normal at a later date. There was no need for thyroid hormone replacement therapy in any of these cases. The non-thyroidal illnesses are summarised in Table 42. There was no firm diagnosis reached in case number 127515 who presented with a two week history of anorexia and dullness. However, this dog responded to non-specific therapy without the need for T_4 replacement therapy.

Case Number	Age (yrs)	Sex	Breed	Basal total T_4 (nmol/L)	Post-TSH total T_4 (nmol/L)	Free T_4 (pmol/L)	Total T_3 (nmol/L)	cTSH (ng/ml)
122647	10.8	M	Boxer	12.4	25.4	10.98	2.61	3.32
129912	11	F	Pug	9.6	21.9	9.46	1.03	0.73
130239	9	FN	Doberman	13.8	22.4	12.37	1.54	0.94

Table 41. Hormone results from three hypothyroid dogs with equivocal TSH response test results.

Case Number	Age (yrs)	Sex	Breed	Diagnosis	Basal Total T_4	Post TSH Total T_4	Free T_4	Total T_3	cTSH
121319	5	M	Chow	Obesity and Musculoskeletal disease	8.5	24.7	ND	ND	0.03
125089	13	FN	WHWT	Allergic Skin Disease	10.8	25.6	33.29	1.46	0.24
127515	10	MN	Golden retriever	Temporary anorexia and lethargy	10.1	23.9	ND	ND	0.18
127978	8	FN	Bearded collie	Addisons Disease	10.5	22.5	8.0	0.99	2.65
129496	11	FN	Boxer	HAC	4.7	20.6	5.81	1.90	0.13
129960	9	MN	Retriever cross	Chronic hyperplastic dermatitis	10.8	22.1	8.95	2.82	0.26
132278	3	MN	Labrador retriever	HAC	12.1	25.2	13.75	1.18	0.28
132320	12	M	Golden retriever	Horner's Syndrome	8.3	24.0	5.19	2.51	0.47
132467	12	M	Crossbreed	Testicular Neoplasia	15.4	23.4	5.0	2.46	0.50

Table 42. Hormone results and clinical details from nine euthyroid dogs with equivocal TSH response test results.

ND Analysis not done HAC Hyperadrenocorticism

WHWT West Highland White Terrier

Appendix 28

Further Investigation of Case Number 133791

Concurrent NTI was confirmed and may have been responsible for "normal" cTSH concentrations in eight of the 11 hypothyroid dogs. Of the remaining three cases the cTSH concentration approached the assay LoD in only one case, a 7.5 year old male entire miniature schnauzer (case number 133791). Further investigation of this case was performed to evaluate the possibility of central hypothyroidism. The initial hormone concentrations from this dog are detailed in Table 43. Routine biochemistry and haematology results are detailed in Table 44.

The pituitary responsiveness to TRH administration was assessed by estimation of circulating cTSH concentration at five, 10, 20, 30 and 60 minutes following the intravenous administration of 200 μg synthetic TRH (p-GLU-HIS-PRO amide, Sigma Chemical Co.) These results are detailed in Table 45. There was no significant change in cTSH concentration following TRH administration. This is consistent with a provisional diagnosis of central hypothyroidism. Alternative explanations include the presence of undetectable isomeric forms of cTSH (Peterson *et al.*, 1997) or long-standing hypothyroidism associated with pituitary "exhaustion" (Scott-Moncrieff *et al.*, 1998). In both these situations no measurable cTSH response to TRH administration would also be expected.

Clinical evaluation did not reveal any neurological abnormalities suggestive of a pituitary mass. Further investigations to confirm central hypothyroidism would have consisted of pituitary imaging techniques such as magnetic resonance imaging (MRI). After considerable discussion with the owners of this case, THRT was instituted without any further diagnostic evaluation. The dog responded well and is reported to be clinically healthy after a follow up period of 22 months.

Case Number	Age	Sex	Basal Total T_4	Post-TSH Total T_4	Free T_4	Total T_3	cTSH
133791	7.5	M	5.1	6.2	3.22	2.57	0.02

Table 43. Routine endocrine results from a 7.5 year old hypothyroid male miniature schnauzer (case number 133791). For key see Appendix 4.

Analyte	Result	Analyte	Result
Urea	6.7	CK	85
Na	141	Trig	5.13
K	4.2	GGT	12
Cl	108	Fruct	324
Ca	2.73	RBC	5.06
Mg	0.76	Hb	12
PO4	1.48	HCT	34
Gluc	5.6	MCV	67
Chol	33.07	MCH	23.7
Crea	88	MCHC	35.2
Tbil	1	PLT	530
ALKP	60	MPV	7
AST	17	WBC	5.7
ALT	76	Band N	0
TP	78	Neut	3.591
Alb	37	Lymp	1.824
Glob	41	Mono	0.228
A:G	0.9	Eosin	0.057

Table 44. Routine biochemistry and haematology results from a 7.5 year old hypothyroid male miniature schnauzer (case number 133791) with an unexpectedly low circulating cTSH concentration. For key and units see Appendices 2 and 4.

Time post TRH (minutes)	cTSH (ng/ml)
0	0.02
5	0.03
10	0.03
20	0.02
30	0.02
60	0.03

Table 45. Endogenous cTSH concentrations immediately prior to and at following the intravenous administration of 200 μg TRH in case number 133791.

Appendix 29

Signalment and hormone data from 52 hypothyroid dogs

Case Number	Age (yrs)	Sex	Breed	Basal Total T_4 (nmol/L)	Post-TSH Total T_4 (nmol/L)	Free T_4 (pmol/L)	Total T_3 (nmol/L)	cTSH (ng/ml)
116865	7.2	M	Scottie	7.6	4.7	3.22	0.81	3.30
131136	6	MN	Cocker sp.	4.7	6.0	3.22	1.33	0.91
131132	11	FN	WHWT	11.4	10.0	5.41	3.88	12.3
131486	10.2	FN	WHWT	14.0	19.6	10.34	1.05	3.25
124413	6	F	Labrador	14.8	11.0	11.78	1.09	1.74
126382	6	M	Sheltie	4.7	4.7	3.22	0.66	1.57
129827	4.5	M	Cocker sp.	4.7	4.7	3.22	1.25	1.89
133137	10	FN	Crossbreed	4.7	4.7	3.22	1.32	3.57
133791	7.5	M	Miniature schnauzer	5.1	6.2	3.22	2.57	0.02
124684	10	FN	G Ret.	5.8	8.7	4.11	0.25	0.94
115099	6.9	F	Bearded collie	10.4	10.5	ND	ND	2.84
130239	9	FN	Doberman	13.8	22.4	12.37	1.54	0.94
109711	9	M	Miniature schnauzer	12.9	19.3	3.22	0.70	0.15
133026	3	M	GSD cross	13.8	15.7	3.22	23.49†	8.52
130794	10	F	Crossbreed	5.2	13.1	3.22	1.47	0.96
129292	5	MN	Akita	10.8	8.0	3.22	1.61	0.43
109116	7	F	Clumber sp	4.7	5.6	ND	ND	1.29
131646	11	FN	Border terrier	4.7	4.7	8.85	0.86	0.73
132396	4	F	G-Ret	12.8	13.7	4.93	2.88	4.34
122647	10.75	M	Boxer	12.4	25.4	10.98	2.61	3.32
134214	8	M	Lab-Ret	5.3	4.7	3.22	2.32	2.10
119760	4	M	Hovawart	9.1	8.5	3.22	1.11	6.31
133399	5	M	Lab-Ret	4.7	4.7	3.22	1.13	1.88
130375	8	FN	Crossbreed	4.7	4.7	3.22	0.91	1.42
127889	9	F	Shetland sheepdog	4.7	4.7	3.22	0.81	2.90
129912	11	F	Pug	9.6	21.9	9.46	1.03	0.73
134156	3	F	Eng sp sp	4.7	4.7	3.22	1.50	1.18
127391	8	FN	Rough collie	4.7	4.7	ND	ND	0.23
129838	4	FN	Eng sp sp	5.6	6.6	3.22	1.35	2.48
130866	10	M	Labrador	4.7	4.7	3.22	1.22	1.68
129976	7	FN	WHWT	4.7	4.7	3.22	1.65	5.98
130656	6	FN	Akita	4.7	13.2	3.22	1.43	0.69

Appendix 29 continued

Case Number	Age (yrs)	Sex	Breed	Basal Total T_4 (nmol/L)	Post-TSH Total T_4 (nmol/L)	Free T_4 (pmol/L)	Total T_3 (nmol/L)	cTSH (ng/ml)
132798	8	M	Border terrier	4.7	5.0	3.22	1.16	0.03
130431	12	F	St Bernard	21.2	29.4	12.39	1.88	0.57
128082	9	FN	Crossbreed	4.7	4.7	3.22	0.96	2.67
127111	7	F	Lab-Ret	5.5	4.7	3.22	1.04	2.17
124283	6	M	Rottweiler	4.7	4.7	ND	ND	1.02
133778	3	M	Bassett hound	4.7	4.7	3.22	2.23	0.08
133641	4	FN	Flat coated retriever	4.7	4.7	3.22	1.77	2.13
125877	4	MN	Crossbreed	4.7	4.7	ND	ND	1.45
132391	13	FN	Doberman	4.8	10.0	3.22	1.59	1.52
127627	9	M	Poodle	4.7	4.7	ND	ND	0.01
134019	11	F	English setter	17.8	18.8	6.66	3.57	6.30
131249	10	F	Cocker sp	4.7	4.7	ND	ND	1.17
131426	9	F	Lab-Ret	4.7	4.7	3.22	1.21	1.77
127135	9.5	M	Retriever	6.4	9.2	ND	ND	0.15
133668	7	M	Hungarian vizsla	4.7	4.7	3.22	1.82	0.26
127940	6	FN	Doberman	4.7	4.7	3.22	1.08	0.98
129565	4	MN	Cocker sp	14.5	17.4	11.90	ND	0.84
135542	9	MN	Labrador cross	4.7	4.7	3.22	1.91	1.34
122424	9.75	M	Rottweiler	4.8	15.2	ND	ND	0.76
131438	7	F	GSP	4.7	4.7	3.22	1.02	0.03

†Elevated result attributed to the presence of T_3Ab.

WHWT	West Highland White Terrier	GSP	German shorthaired pointer
GSD	German Shepherd Dog	Eng sp sp	English springer Spaniel
Cocker sp	Cocker Spaniel	Clumber sp	Clumber Spaniel
G-Ret	Golden retriever	Lab-Ret	Labrador retriever
ND	Analysis not done		

Appendix 30

Signalment and hormone data from 88 euthyroid dogs with non-thyroidal illness

Case Number	Age (yrs)	Sex	Breed	Basal Total T_4 (nmol/L)	Post-TSH Total T_4 (nmol/L)	Free T_4 (pmol/L)	Total T_3 (nmol/L)	cTSH (ng/ml)
124709	10	M	Crossbreed	16.9	63.9	12.04	2.62	0.16
132320	12	M	Golden retriever	8.3	24.0	5.19	2.51	0.47
130370	2	M	Bull terrier	32.1	63.9	13.70	3.09	0.66
132582	7	M	Dachshund	15.2	46.5	13.71	ND	1.00
130929	10.5	M	PMD	10.7	46.0	7.51	1.90	0.12
132476	12	M	Crossbreed	15.4	23.4	5.00	2.46	0.50
124980	7.5	F	Gordon setter	4.90	37.3	4.68	0.94	0.08
131778	4	M	Bull mastiff	19.3	34.4	9.99	2.37	0.06
132904	9	MN	Cocker spaniel	23.7	73.3	11.28	2.94	0.72
130754	11	F	Golden retriever	27.1	63.8	12.00	2.68	0.18
126582	5.5	M	Golden retriever	21.6	72.4	31.44	1.54	0.06
127405	5	FN	Golden retriever	29.5	51.7	17.88	2.47	0.22
122827	2.5	FN	Shitsu	20.4	50.3	24.74	1.47	0.09
131049	9.5	FN	Crossbreed	18.7	75.3	9.96	2.96	0.11
127416	11	M	Sheltie	28.9	85.7	ND	ND	0.34
131930	10.25	M	Labrador	18.8	46.8	11.03	2.27	0.44
130820	1.5	M	BMD	12.6	55.5	6.75	ND	0.18
124776	7	FN	Boxer	23.3	45.8	28.46	1.86	0.21
134192	9	M	Rottweiler	13.5	39.3	10.49	2.53	0.57
131408	7	M	Labrador retriever	8.2	31.2	9.75	2.03	0.24
130143	8	M	Eng sp sp	19.4	66.9	13.74	3.01	0.87
128293	5	F	Rotty	23.7	46.3	22.46	1.31	0.07
121319	5	M	Chow chow	8.5	24.7	ND	ND	0.03
131579	10	MN	Border collie	14.8	67.7	12.01	2.25	0.67
131484	1.5	FN	Bichon frise	60.5	187.6	35.44	3.11	0.12
119621	7	F	Bearded collie	26.0	45.1	10.73	3.31	1.37
130680	7.5	M	Bearded collie	21.4	75.1	13.10	ND	0.87
124358	2	M	Labrador-retriever	27.5	45.0	21.00	1.93	0.32
130075	3	M	Chow-chow	23.0	72.9	20.86	2.43	0.02
132502	10	M	Golden retriever	17.8	57.8	15.41	1.78	0.07
129205	14	FN	Pekinese	16.3	37.8	19.54	1.26	0.28
127911	5	M	Dachshund	21.0	54.0	23.7	1.80	0.14
127163	12	M	Collie cross	29.6	53.7	32.34	0.78	0.01
129960	9	MN	Retriever cross	10.8	22.1	8.95	2.82	0.26
126068	9	M	Doberman	11.0	42.9	16.06	1.82	0.31
110826	10	F	Labrador retriever	15.2	31.5	11.50	2.38	0.45
132446	10	FN	Crossbreed	4.7	34.7	3.22	2.71	0.02

Appendix 30 continued

Case Number	Age (yrs)	Sex	Breed	Basal Total T_4 (nmol/L)	Post-TSH Total T_4 (nmol/L)	Free T_4 (pmol/L)	Total T_3 (nmol/L)	cTSH (ng/ml)
130040	6	FN	CKCS	18.4	77.7	15.19	3.02	0.04
126932	7.5	FN	Irish Setter	27.5	70.9	23.83	1.93	0.27
134203	5	F	Yorkshire terrier	49.8	127	ND	ND	0.17
134197	1.5	FN	Golden retriever	17.8	67.2	8.29	2.58	0.10
131217	2	M	Dalmation	31.1	85.6	20.16	2.65	0.54
126385	5	FN	Labrador	26.6	52.7	32.78	1.16	0.37
130364	6.5	M	Irish setter	10.1	34.6	7.49	1.27	0.29
132278	3	MN	Labrador	12.1	25.2	13.75	1.18	0.28
131932	5	MN	Boxer	33.0	72.0	16.17	3.27	0.95
131280	13	M	Wire haired fox terrier	27.6	86.1	14.83	2.69	0.19
130350	4	FN	Bull terrier cross	41.4	113.4	15.35	4.71	0.51
131783	3	M	CKCS	21.3	190	8.00	3.24	0.02
133497	9.5	M	Border collie	13.4	80.2	26.94	1.85	0.88
133293	10	F	GSD	8.3	31.3	ND	ND	1.13
129581	5	MN	Doberman	21.9	45.5	11.88	2.19	0.90
127515	10	MN	Retriever	10.1	23.9	ND		0.18
131428	11	M	Golden retriever	13.8	40.3	11.01	2.12	0.50
127978	8	FN	Bearded collie	10.5	22.5	8.00	0.99	2.65
129496	11	FN	Boxer	4.7	20.6	5.81	1.90	0.13
130120	12	FN	Scottie	20.0	35.0	13.94	2.91	0.61
127729	4.5	M	Bull terrier	25.6	45.9	22.16	2.78	1.04
130021	9	F	Sheltie	27.4	130.8	24.25	3.10	0.07
130963	4	F	Doberman	20.2	53.0	9.00	2.33	0.68
132168	8	F	Jack russell terrier	50.8	121.4	24.43	3.22	0.31
124329	2	M	Golden retriever	13.9	33.8	ND	ND	0.30
130849	6	F	Crossbreed	31.4	164.6	14.45	2.07	0.14
132239	11.5	FN	Cocker spaniel	19.3	47.8	12.66	1.93	0.53
124911	4.5	F	Gordon setter	35.0	83.0	51.25	1.41	0.37
131528	11	F	Border collie	17.8	50.0	17.52	2.19	0.43
132510	10	FN	Golden retriever	12.8	32.3	10.2	ND	0.02
131182	7	F	Belgian shepherd	54.4	82.2	11.44	4.59	0.29
131258	8	F	Borzoi	11.7	36.7	9.01	2.72	0.80
130406	7	M	Flat coated retriever	17.3	51.5	12.29	2.86	1.63
131785	4	M	Eng SpSp	36.1	70.5	15.50	2.82	0.18
131709	11	MN	Lhasa apso	25.4	97.4	23.56	2.34	0.23
133780	9.5	MN	Cairn terrier	34.9	94.7	16.49	2.68	0.17
131288	4	FN	Lhasa apso	26.6	76.7	15.34	2.56	0.16
124766	3.5	F	Min schnauzer	46.5	100.8	19.99	3.38	0.20
132385	14.5	M	Labrador	9.8	32.4	4.39	2.92	0.13
126035	1	M	Scottish terrier	33.0	65.0	31.21	1.26	0.10
129797	5	M	Golden retriever	10.2	52.3	10.64	1.52	0.03
129161	4	M	Golden retriever	27.5	63.3	17.85	2.67	0.05

Appendix 30 continued

Case Number	Age (yrs)	Sex	Breed	Basal Total T_4 (nmol/L)	Post-TSH Total T_4 (nmol/L)	Free T_4 (pmol/L)	Total T_3 (nmol/L)	cTSH (ng/ml)
125089	13	FN	WHWT	10.8	25.6	33.29	1.46	0.24
127258	11	M	English setter	22.2	58.2	41.75	1.82	0.34
125949	7	FN	Rottweiler	23.2	67.2	18.40	1.95	0.17
130548	11	MN	Doberman	13.1	48.7	7.77	2.63	0.89
117757	5.25	M	Newfoundland	35.9	59.1	22.64	1.38	0.17
131276	7	FN	Samoyed	28.4	63.9	10.59	2.93	2.10
130897	6	F	Cairn terrier	15.7	45.5	11.40	2.24	0.77
132249	7	M	WHWT	19.3	59.2	16.12	2.80	0.37
126513	5	FN	Bearded collie	29.6	63.7	23.27	ND	0.47

WHWT	West Highland White Terrier	BMD	Bernese Mountain Dog
GSD	German Shepherd Dog	PMD	Pyrenean Mountain Dog
CKCS	Cavalier King Charles Spaniel	ND	Analysis not done

Appendix 31

Analysis of breed and specialty distributions within the hospital control group.

Dogs within the hospital control group were grouped according to the specialty to which they were referred. The distribution of breeds within these groups were then determined and are detailed in Table 46. A selection of these data are displayed in Figure 30. The hospital computer breed coding used for case grouping is detailed in Table 47.

As can be appreciated from Table 46 and Figure 30 a small number of disciplines accounted for a relatively large percentage of the total referral group. The most common disciplines to which cases were referred were general medicine (n=1047, 18.2 %), orthopaedics (n=829, 14.4 %), ophthalmology (n=828, 14.4 %) and soft tissue surgery (n=691, 12.0 %). The specialties which accounted for the largest relative referrals of individual breeds are detailed in Table 48. It can be seen from these data that certain breeds were more commonly referred to particular specialties. For example 20 of 50 (40.0 %) daschunds and 12 of 27 Pekinese (44.4 %) were referred to neurology, and 27 of 99 (27.3 %) poodles were referred to ophthalmology. These biases are unlikely to significantly damage the control data if the actual number of animals of each breed involved remains small in relation to the total number of control cases.

In addition to evaluating the percentage of each breed referred to each specialty, the percentage of each specialty accounted for by individual breeds was examined. The specialties in which an individual breed accounted for greater than 15 % of the total referrals to that specialty are detailed in Table 49. It can be seen from these data that certain specialties were composed predominantly of one or two breeds of dog. For example, of the 69 cases within the pathology specialty, 66 (95.6 %) were German shepherd dogs. This is potentially much more damaging to the validity of the control group due to the introduction of a large number of a particular breed. Closer investigation of the pathology groups revealed that these were dogs actively recruited to the University of Glasgow Veterinary School for a particular study on chronic degenerative radiculomyelopathy (CDRM). The categorisation of breeds and specialties in this manner allowed the identification of such biases.

From the data presented it is clear that there is the potential for certain breeds referred in disproportionately high numbers to particular specialties, to influence the

makeup of the hospital control group as a whole. Prior to interrogating the database, it had been the intention to statistically analyse the distribution of breeds within specialties by chi squared analysis. This would have allowed identification and removal of offending subsets of dogs. However, as is illustrated by the data in this appendix, multiple breeds across multiple disciplines were present in sufficiently large numbers to make statistical evaluation of the data extremely complicated. In addition, chi squared analysis became inappropriate since the appropriate R x C contingency tables had numerous cells which were either empty or below a value considered statistically safe (usually five) for analysis.

An alternative approach which was considered, was to compare the distribution of breeds within a specialty, to the distribution of that breed within all other specialties as a single group. This approach would have allowed simple chi squared analysis and has been applied previously in a similar situation (Graham, 1995). However, after consideration that approach was rejected since any overrepresentation of a breed within a discipline would in itself, when included in the control group, reduce the likelihood of identifying over-referral of that breed within any other specialty. The detail involved in analysing the hospital group for the purpose of producing a valid control was consequently considered beyond the scope of this thesis. The hospital group was subsequently used unaltered as the control. It was felt that its comparative value with other studies who have used equivalent control cases, alongside recognition of the inherent problems associated with this approach justified its inclusion in this study.

Appendix 31 (continued)

Breed	Cardiology	Dermatology	Endocrinology	Neurology	Oncology	Ophth	Ortho	Path	Repro	General Medicine	Soft Tissue	Others	Total
1	0	0	0	0	0	0	1	0	0	1	0	0	2
2	0	2	1	0	0	3	6	0	0	6	1	2	21
3	0	1	0	0	0	0	1	0	0	0	0	0	2
4	0	1	0	0	0	0	0	0	0	1	0	0	2
5	1	1	0	3	2	2	3	0	2	7	2	4	27
6	0	0	1	1	0	0	0	0	0	0	0	1	3
7	3	0	3	5	1	2	12	0	1	6	2	5	40
8	3	3	2	2	0	3	1	0	0	3	1	0	18
9	0	0	0	0	0	0	1	0	0	0	0	0	1
10	0	0	0	0	1	0	0	0	0	0	0	0	1
11	0	1	0	0	0	0	0	0	0	0	1	0	2
12	33	16	9	15	7	22	17	1	6	28	19	5	178
13	0	0	0	0	0	0	3	0	0	0	0	0	3
14	1	3	0	0	2	6	3	0	3	2	6	3	29
15	2	2	2	3	4	5	12	0	6	8	7	5	56
17	3	0	0	3	0	3	2	0	0	1	1	2	15
19	2	3	1	1	0	5	4	0	2	6	4	0	28
20	22	28	36	28	26	55	85	0	6	88	88	27	489
22	1	1	1	2	2	1	2	0	1	6	1	0	18
23	4	3	2	20	0	2	3	0	0	12	1	3	50
24	5	3	1	4	0	3	3	0	1	3	4	1	28
25	2	1	1	9	2	0	0	0	0	4	0	0	19
26	4	4	7	19	4	4	7	0	1	30	8	8	96
27	0	0	0	0	0	1	1	0	0	1	1	0	4
31	0	0	0	0	0	0	0	0	0	0	2	0	2
32	20	40	18	25	13	35	64	66	5	95	86	17	484
33	7	4	4	11	1	10	12	0	4	13	1	4	71
34	4	3	1	2	2	0	8	0	5	20	2	4	51
35	0	0	1	0	0	2	0	0	0	1	0	0	4
38	0	0	0	0	0	1	0	0	0	0	0	0	1

Appendix 31 (continued)

Breed	Cardiology	Dermatology	Endocrinology	Neurology	Oncology	Ophth	Ortho	Path	Repro	General Medicine	Soft Tissue	Others	Total
39	9	5	2	7	1	0	5	0	0	10	3	1	43
41	1	1	0	0	0	1	2	0	0	5	1	0	11
43	0	0	0	0	0	3	0	0	0	2	0	0	5
45	1	0	0	0	0	1	1	0	0	1	1	0	5
48	0	0	0	0	0	0	0	0	0	0	0	1	1
49	0	0	0	0	0	0	1	0	0	0	0	0	1
50	0	4	3	3	0	9	2	0	1	4	2	3	31
51	0	0	0	0	0	0	0	0	0	0	1	0	1
53	3	0	1	4	2	4	3	0	0	2	5	0	24
54	0	0	0	1	0	0	0	0	1	2	1	1	6
55	2	1	1	2	0	3	0	0	0	3	1	3	16
57	0	1	0	0	0	1	5	0	2	2	1	3	15
59	1	1	0	0	0	0	0	0	0	0	0	0	2
60	3	2	1	7	0	4	14	0	0	9	1	2	43
62	1	0	0	1	1	1	1	0	4	7	9	2	27
63	1	1	0	1	0	0	6	0	0	1	2	0	12
64	1	2	1	12	0	4	4	0	0	2	1	0	27
65	0	0	0	1	0	0	0	0	0	0	0	0	1
66	7	5	1	4	0	3	7	0	1	2	6	1	37
67	3	1	0	2	0	3	2	0	1	0	2	0	14
68	5	4	8	10	5	27	12	0	1	11	10	6	99
70	1	0	1	0	0	0	0	0	0	1	2	0	5
71	0	0	0	0	0	1	1	0	0	2	1	0	5
73	2	2	0	1	0	1	4	0	0	1	1	0	12
74	64	66	46	55	69	139	200	0	19	198	103	56	1015
75	1	2	2	0	1	2	4	0	0	5	1	2	20
76	4	1	10	12	12	12	30	0	5	35	14	8	143
77	4	0	0	3	2	0	9	0	1	9	2	2	32
79	2	1	4	0	4	8	2	0	0	6	2	2	31
80	0	0	1	1	0	0	0	0	0	0	0	0	2

Appendix 31 (continued)

Breed	Cardiology	Dermatology	Endocrinology	Neurology	Oncology	Ophth	Ortho	Path	Repro	General Medicine	Soft Tissue	Others	Total
81	3	5	5	5	0	6	1	0	0	5	4	3	37
83	9	4	2	5	2	11	10	0	0	14	15	7	79
84	1	4	2	3	2	9	8	0	0	13	5	2	49
85	2	4	1	3	1	9	3	0	2	3	2	1	31
87	55	53	29	58	27	127	69	0	6	100	64	44	632
88	0	1	0	0	0	1	2	0	0	2	1	0	7
89	0	0	0	0	0	1	1	0	0	0	0	1	3
92	44	100	57	56	34	190	103	0	26	155	111	65	941
94	0	0	1	2	0	0	2	0	1	1	1	2	10
95	0	0	1	2	2	0	3	0	3	4	1	0	16
96	1	4	4	4	2	5	8	0	2	10	5	2	47
97	0	1	2	2	0	1	1	0	1	5	2	1	16
98	1	3	0	0	0	9	1	0	0	0	1	1	16
99	19	36	31	39	28	67	51	2	9	73	68	24	447
Total	368	435	308	459	262	828	829	69	129	1047	691	337	5762

Table 46. Distribution of breeds of dogs within each referral discipline in the hospital group.

For explanation of breed codes see Table 47.

Repro Reproduction Ortho- Orthopaedics Ophth Ophthalmology

Appendix 31 (continued)

Code	Breed	Code	Breed	Code	Breed	Code	Breed
1	Affenpinscher	26	Doberman	51	Lowchen	76	Rottweiler
2	Akita	27	Elkhound	52	Lundehund	77	St Bernard
3	Anatolian karabash	28	Estrela Mountain Dog	53	Lurcher	78	Saluki
4	Basenji	29	Fila Brasiliero	54	Malamute	79	Samoyed
5	Beagle	30	Foxhound	55	Maltese	80	Schipperke
6	Belgian Shepherd	31	French Bulldog	56	Maremma Sheepdog	81	Schnauzer
7	Bernese Mountain Dog	32	German Shepherd Dog	57	Mastiff	82	Sennenhund
8	Bichon Friese	33	Great Dane	58	Mexican hairless	83	Setter
9	Bloodhound	34	Greyhound	59	Munsterlander	84	Sheltie
10	Borzoi	35	Griffon	60	Newfoundland	85	Shih Tzu
11	Bouvier des Flandres	36	Hamilton Stovare	61	Norwegian Buhund	86	Sloughi
12	Boxer	37	Harrier	62	Old English Sheepdog	87	Spaniel
13	Briard	38	Lancashire Heeler	63	Papillon	88	Spinone
14	Bulldog	39	Lancashire Heeler	64	Pekinese	89	Spitz
15	Bullmastiff	40	Hovawart	65	Pinscher	90	Staghound
16	Canaan Dog	41	Husky	66	Pointer	91	Swedish Vallhund
17	Chihuahua	42	Italian Greyhound	67	Pomeranian	92	Terrier
18	Chinese Crested	43	Japanese Chin	68	Poodle	93	Tosa
19	Chow Chow	44	Karelian Bear Dog	69	Portugese Water Dog	94	Trailhound
20	Collie	45	Keeshond	70	Pug	95	Viszla
21	Coonhound	46	Kelpie	71	Puli Hungarian	96	Weimeraner
22	Corgi	47	Komondor	72	Pumi	97	Whippet
23	Dachshund	48	Kuvasz	73	Pyrenean Mountain Dog	98	Sharpei
24	Dalmation	49	Leonberger	74	Retrievers	99	Crossbreed
25	Deerhound	50	Lhasa Apso	75	Rhodesian Ridgeback		

Table 47. Hospital computer codes used to categorise individual breeds

Appendix 31 (continued)

Specialty	Breed	Number of dogs	Percentage of total referrals of that breed
Cardiology	Boxer	33	18.5 %
Dermatology	Terriers	100	10.6 %
General Medicine	Retrievers	198	19.5 %
	Terriers	155	16.5 %
	Spaniels	100	15.8 %
	GSD	95	19.6 %
	Collies	88	18.0 %
	Crossbreeds	73	16.3 %
	Rottweiler	35	24.5 %
	Doberman	30	31.2 %
	Boxer	28	15.7 %
	Greyhound	20	39.2 %
	Setters	14	17.7 %
	Great Dane	13	18.3 %
	Shetland Sheepdogs	13	26.5 %
	Dachshunds	12	24.0 %
	Poodles	11	11.1 %
Neurology	Dachshund	20	40.0 %
	Doberman	19	19.8 %
	Pekinese	12	44.4 %
	Great Dane	11	15.5 %
Ophthalmology	Terriers	190	20.2 %
	Retrievers	139	13.7 %
	Spaniels	127	20.1 %
	Crossbreeds	67	15.0 %
	Collies	55	11.2 %
	Poodles	27	27.3 %
	Boxer	22	12.4 %
	Tosa	11	13.9 %

Table 48.

Appendix 31 (continued)

Specialty	Breed	Number of dogs	Percentage of total referrals of that breed
Orthopaedics	Retrievers	200	19.7 %
	Terriers	103	10.9 %
	Collies	85	17.4 %
	Spaniels	69	10.9 %
	GSD	64	13.2 %
	Crossbreeds	51	11.4 %
	Rottweiler	30	21.0 %
	Newfoundland	14	32.6 %
	Bernese Mountain Dog	12	30.0 %
	Bullmastiff	12	21.4 %
	Great Dane	12	16.9 %
	Poodle	12	12.1 %
Pathology	GSD	66	13.6 %
Soft Tissue Surgery	Terriers	111	11.8 %
	Retrievers	103	10.1 %
	Collies	88	18.0 %
	GSD	86	17.8 %
	Crossbreeds	68	15.2 %
	Spaniels	64	10.1 %
	Boxer	19	10.7 %
	Setters	15	19.0 %

Table 48 (continued) Specialties in which individual breeds constituted more than 10 percent of the total referrals of that breed (only groups with more than 10 individuals are included)

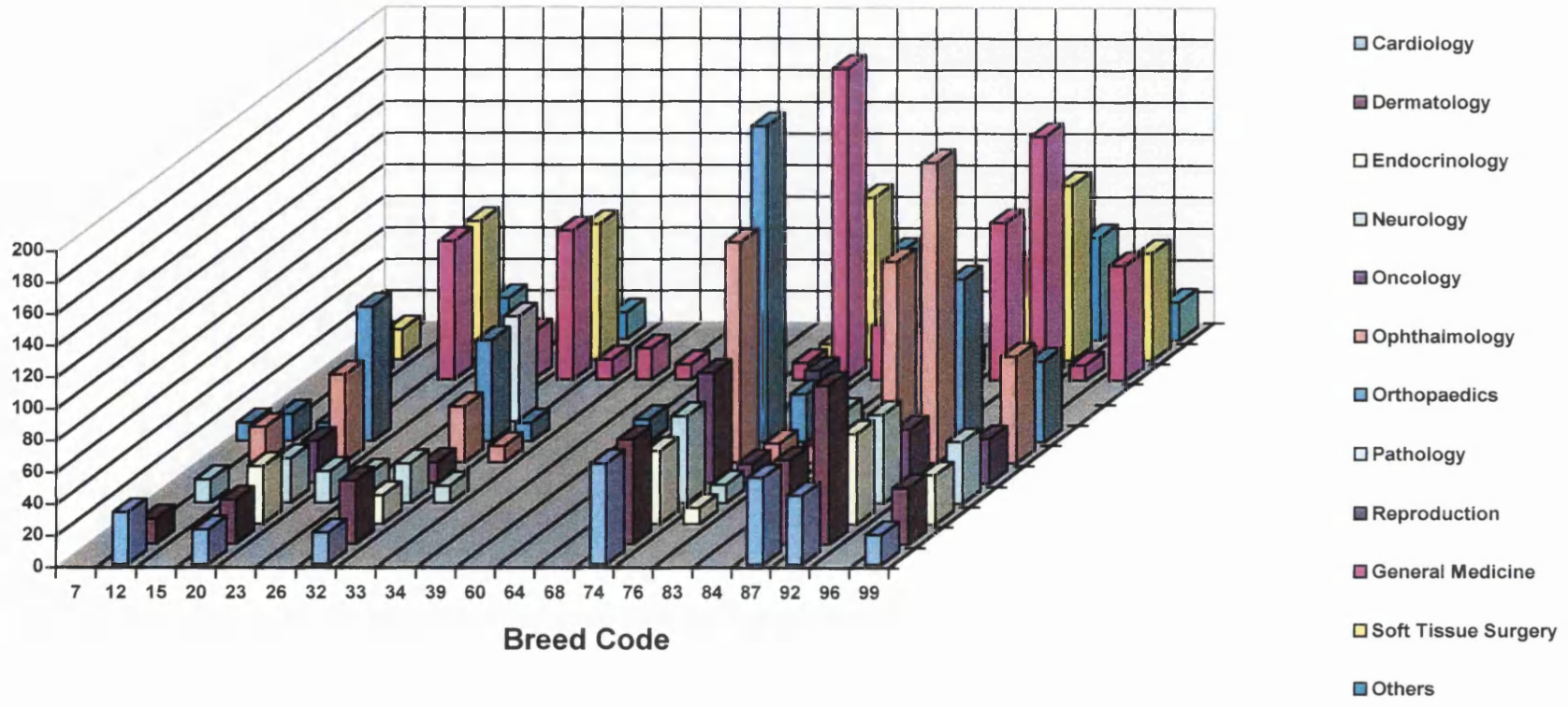


Figure 30. Breakdown of the hospital referral group displaying the number of dogs of each breed within each referral specialty. (Only values greater than 10 are displayed)

Appendix 31 (continued)

Specialty	Breed	Number of dogs	Percentage of total referrals to that specialty accounted for by that breed
Cardiology	Retrievers	64	17.4 %
Dermatology	Terriers	100	23.0 %
	Retrievers	66	15.2 %
Endocrinology	Terriers	57	18.5 %
General Medicine	Retrievers	198	18.9 %
Oncology	Retrievers	69	26.3 %
Ophthalmology	Terriers	190	22.9 %
	Retrievers	139	16.8 %
	Spaniels	127	15.3 %
Orthopaedics	Retrievers	200	24.1 %
Pathology	GSD	66	95.6 %
Reproduction	Terriers	26	20.2 %
Soft Tissue Surgery	Terriers	111	16.1 %
Others	Terriers	65	19.3 %
	Retrievers	56	16.7 %

Table 49. Breeds of dog within each referral specialty in which the particular breed represented more than 15 % of the total referrals to that specialty.

Appendix 32

Further evaluation of case number 127627. A case with concurrent hypothyroidism and hyperadrenocorticism

A nine year old entire male poodle was referred with a history of lethargy, flank alopecia, superficial pyoderma, abdominal distention and more recently, polydipsia and polyphagia. Subsequently, neurological abnormalities developed, most notably right peripheral vestibular syndrome and bilateral facial nerve paralysis. Abnormal routine and endocrine clinicopathological findings including results of ACTH stimulation and low dose dexamethasone suppression testing are detailed in Tables 50 and 51. Based on the clinical and clinicopathological findings, a diagnosis of pituitary dependent hyperadrenocorticism (PDH) was made.

Analyte	Result	Comment
Cholesterol (mmol/L)	17.81	Increased
ALKP (i.u/L)	1774	Increased
ALT (i.u./L)	256	Increased
Lymphocytes (x 10^9/L)	0.889	Decreased

Table 50. Routine clinicopathological abnormalities in case number 127627.

Test	Time	Result	Comment
ACTH Stimulation	Pre ACTH cortisol	130 nmol/L	Consistent with
	Post ACTH cortisol	670 nmol/L	hyperadrenocorticism
Low Dose	Basal cortisol	121 nmol/L	Consistent with
Dexamethasone	Three Hours post ACTH	9 nmol/L	pituitary dependent
Suppression	Eight Hours post ACTH	37 nmol/L	hyperadrenocorticism
	Basal Total T_4	LoD	Consistent with
TSH stimulation	Six Hours post TSH	LoD	hypothyroidism but with
	Basal cTSH	0.01 ng/ml	discordant cTSH

Table 51. Dynamic endocrine test results in case number 127627.

LoD: result at or below the assay limit of detection

Due to the suppressive effect of NTI on thyroid function, the diagnosis of hypothyroidism remained provisional and thyroid function was monitored following treatment for hyperadrenocorticism. Mitotane treatment was commenced and resulted in partial resolution of the clinicopathological and clinical abnormalities within 10 days. However the metabolic clinical signs, and total T_4 concentration in particular remained abnormal despite otherwise adequate therapy confirmed by monitoring ACTH stimulation tests (Table 52). Following six weeks of mitotane therapy there was no change in thyroid hormone parameters and a diagnosis of concurrent hypothyroidism was made.

Date	Total T_4 (nmol/L)	cTSH (ng/ml)	Basal cortisol (nmol/L)	Post-ACTH cortisol (nmol/L)	Comment
26/3/97	LoD	LoD	130	670	Therapy for hyperadrenocorticism starts
1/4/97	LoD (pre) LoD (post)	LoD			TSH stimulation test
10/4/97	4.9	0.03	LoD	LoD	
17/4/97	LoD	LoD			
13/5/97	LoD	LoD			No change in thyroid status

Table 52. Endocrine results with time in case 127627.

LoD: result at or below the assay limit of detection

Since the cTSH concentration in this dog remained at the cTSH assay LoD, a TRH stimulation test was performed the results of which are detailed in Table 53. These were consistent with a diagnosis of central hypothyroidism.

Time	cTSH (ng/ml)	Comment
0 minutes	0.03	No response to TRH
10 minutes	0.02	administration consistent
20 minutes	0.03	with central
30 minutes	0.02	hypothyroidism

Table 53. Response of cTSH to exogenous TRH administration in case number 127627.

Following the start of thyroid hormone replacement therapy, there was a good clinical response associated with appropriate therapeutic concentrations of the hormone (results not shown). To minimise the interpretation of clinical signs resulting from the hyperadrenocorticism as being due to hypothyroidism only those signs which either developed or persisted following therapy for the hyperadrenocorticism were used in the analysis of clinical signs associated with hypothyroidism outlined in Chapter 5.

Appendix 33

Endocrinological case details recorded from 31 dogs receiving thyroid hormone replacement therapy.

Dog	Hospital number	Date	Days on therapy	Visit	Weight (kg)	Percent starting weight	Total T_4	cTSH
1	116865	17-06-96	0	1			7.6	3.3
1	116865	16-07-96	29	3			56.4	0.01
1	116865	16-09-96	91	4			41.7	0.1
2	131136	23-02-98	0	1	22.5	100	4.7	0.91
2	131136	12-03-98	17	2	21.4	95.1	50	0.07
2	131136	23-04-98	59	3	19.4	86.2	38.4	0.03
2	131136	15-06-98	112	4	20.2	89.8	67.9	0.01
3	131132	06-01-97	0	1	9.8	100	11.4	12.3
3	131132	27-01-97	21	2	9.8	100	13.3	1.65
3	131132	03-03-97	56	3	9.4	95.9	63.7	0.01
3	131132	15-04-97	99	4	9.2	93.9	60.7	0.01
4	131486	03-07-97	0	1	7.3	100	14	3.25
4	131486	04-08-97	32	3	7.5	102.7	26.8	0.54
4	131486	18-09-97	77	4	7.5	102.7	48.4	0.51
5	129827	03-06-96	0	1	22	100	4.7	1.89
5	129827	12-06-96	9	2			62.6	0.02
5	129827	11-07-96	38	3	20.2	91.8	43.5	0.01
5	129827	23-09-96	112	4			53.6	0.02
6	133137	16-10-97	0	1			4.7	3.57
6	133137	30-10-97	14	2			95.1	0.02
6	133137	27-11-97	42	3			52.9	0.02
6	133137	05-02-98	112	4			64.6	0.01
7	133791	20-01-98	0	1	10	100	5.1	0.02
7	133791	09-02-98	20	2	9.1	91	39.6	0.01
7	133791	12-03-98	51	3	9.1	91	37	0.01
8	133026	02-10-97	0	1	55	100	13.8	8.52
8	133026	27-11-97	56	3	48	87.3	58.3	0.32
8	133026	08-01-98	98	4	50.5	91.8	49.2	0.18
9	130794	11-11-96	0	1	32	100	5.2	0.96
9	130794	25-11-96	14	2	32.5	101.6	100.5	0.05
9	130794	24-12-96	43	3	31.7	99.1	77.2	0.05
9	130794	03-02-97	84	4	31.2	97.5	116.5	0.04
10	132396	16-07-97	0	1	33.2	100	12.8	4.34
10	132396	04-08-97	19	2	31.5	94.9	81.1	0.01
10	132396	11-09-97	57	3	31	93.4	103.5	0.01
10	132396	13-10-97	89	4	30	90.4	12.6	0.01
11	122647	24-07-97	0	1	37	100	12.4	3.32
11	122647	08-08-97	15	2			42.3	0.14
11	122647	25-09-97	63	3	36	97.3	42.6	1.62
11	122647	06-11-97	105	4	34.4	93.0	39.9	0.25
12	134214	30-03-98	0	1	39.25	100	5.3	2.1
12	134214	23-04-98	24	2	38.5	98.1	74.6	0.1
12	134214	21-05-98	52	3	36.1	92.0	76	0.02
13	130375	07-10-96	0	1	16	100	4.7	1.42
13	130375	21-10-96	14	2	14.4	90	54.2	0.31
13	130375	02-12-96	56	3	14.1	88.1	49.2	0.39
13	130375	20-01-97	105	4	14.5	90.6	50.7	1

Appendix 33 (continued)

Dog	Hospital number	Date	Days on therapy	Visit	Weight (kg)	Percent starting weight	Total T_4	cTSH
14	134156	09-03-98	0	1	21.5	100	4.7	1.18
14	134156	26-03-98	17	2	20.5	95.3	52	0.25
14	134156	22-04-98	44	3	20.9	97.2	45	0.19
14	134156	01-06-98	84	4	19.5	90.7	31.4	0.15
15	129838	03-06-96	0	1	23.5	100	5.6	2.48
15	129838	01-07-96	28	2			81.1	0.01
15	129838	19-08-96	77	4	22	93.6	60.8	0.02
16	130866	18-11-96	0	1	46	1	4.7	1.68
16	130866	05-12-96	17	2	44	95.6	52.1	0.15
16	130866	06-01-97	49	3	40.5	88	46.7	0.02
16	130866	17-02-97	91	4	39.3	85.4	51.1	0.03
17	129976	20-06-96	0	1	11.8	100	4.7	5.98
17	129976	16-07-96	26	2	11.3	95.8	76.6	0.02
17	129976	26-08-96	67	3	11	93.2	86.1	0.05
17	129976	07-10-96	109	4	10.7	90.7	62.7	0.06
18	130656	22-10-96	0	1	36	100	4.7	0.69
18	130656	30-12-96	69	3	34.5	95.8	54.2	0.05
18	130656	27-01-97	97	4	33.5	93	50.8	0.01
19	132798	03-09-97	0	1	9.5	100	4.7	0.03
19	132798	12-09-97	9	2	8.9	93.7	47.4	0.01
20	128082	05-03-96	0	1	22.4	100	4.7	2.67
20	128082	29-04-96	55	3	23	102.7	42.3	0.2
21	127111	04-06-96	0	1	32	100	5.5	2.17
21	127111	17-06-96	13	2	33.5	104.7	38.7	0.07
21	127111	09-09-96	97	4	30.5	95.3	95.9	0.19
22	133778	19-01-98	0	1	22.7	100	4.7	0.08
22	133778	05-02-98	17	2	22.5	99.1	79.3	0.03
22	133778	09-03-98	49	3	22.8	100.4	39.8	0.01
22	133778	16-04-98	87	4	22	96.9	71.1	0.01
23	133641	29-12-97	0	1	30.5	100	4.7	2.13
23	133641	12-01-98	14	2	28	91.8	29.3	0.75
23	133641	09-02-98	42	3	29.4	96.4	32	0.01
23	133641	23-03-98	84	4	27.1	88.8	27.7	0.01
24	132391	03-07-97	0	1	30	100	4.8	1.52
24	132391	24-07-97	21	2	27.5	91.7	8.3	0.02
24	132391	01-09-97	60	3	27.5	91.7	41.9	0.01
24	132391	02-10-97	91	4	26.3	87.7	40.9	0.01
25	127627	13-05-97	0	1			4.7	0.01
25	127627	06-06-97	24	2			29.1	0.01
26	134019	18-02-98	0	1			17.8	6.3
26	134019	11-03-98	21	2			26.3	0.02
27	131426	13-02-97	0	1	38.7	1	4.7	1.77
27	131426	27-02-97	14	2	37.2	96.1	90.3	0.04
27	131426	18-03-97	33	3	35.3	91.2	38.5	0.05
27	131426	02-06-97	109	4	29.5	76.2	57.2	0.01
28	127940	30-04-96	0	1	31.5	100	4.7	0.98
28	127970	22-05-96	22	2	31	98.4	36.8	0.1
28	127940	18-06-96	49	3			60.7	0.09
29	129565	07-05-96	0	1	24.2	100	14.5	0.84
29	129565	28-05-96	21	2	22	90.9	53.8	0.03
29	129565	15-08-96	100	4	21	86.8	88	0.03
30	129757	07-11-97	0	1			4.7	1.34
30	129757	21-11-97	14	2			39.4	0.66
30	129757	11-12-97	34	3			31.4	0.22
31	131438	17-02-97	0	1	32.8	100	4.7	0.03
31	131438	06-03-97	17	2	31.5	96.0	67.3	0.01
31	131438	07-04-97	49	3	30	91.5	49.9	0.01

Appendix 34

Biochemical data recorded from 31 dogs receiving thyroid hormone replacement therapy.

Dog	Visit	urea	Na	K	Cl	Ca	Mg	PO4	Glu	Chol	Crea	TBil	ALKP	AST	ALT	T P	Alb	Glob	A:G	CK	Trig	GGt	Fruct
1	1	1.7	141	4	106	2.46	0.6	1.13	6	6.6	69	2	387	16	22	86	28	58	0.48	158	0.37	3	209
1	3									3.08										158	0.37	2	172
1	4	3	143	4	111	2.31	0.66	1.33	6	3.59	61	1	211	21	23	69	29	40	0.72	42	0.56	5	182
2	1	5.6	145	4.8	110	2.81	0.9	1.18	4.9	9.53	111	1	94	22	35	73	38	35	1.09	65	1.7	7	327
2	2	4.5	148	4.5	110	2.65	0.85	1.41	5.1	4.55	63	1	132	20	48	64	37	27	1.37	73	0.81	5	312
2	3	5.1	146	4.4	115	2.55	0.71	1.71	5	4.21	60	1	157	24	43	59	31	28	1.11	91	0.8	4	236
2	4	5.2	145	4.5	113	2.52		1.45	3.9	3.7	58	2	175	20	58	62	35	27	1.30	104	1.1	6	267
3	1	5.4	148	5	131	2.9	0.6	2.07	10.9	8.83	83	0	1544	20	14	106	45	61	0.74	65	16.3	18	351
3	2	4.9	144	4.6	106	2.87	0.73	1.82	7	5.62	92	1	871	28	27	72	41	31	1.32	166	1.6	1	296
3	3	6.1	146	4.4	110	2.88	0.89	1.86	5.8	4.6	107	0	778	18	29	73	39	34	1.15	84	1.98	1	290
3	4	5.7	149	4.6	112	2.93	0.86	2.02	6.1	5.32	76	0	827	27	29	73	42	31	1.35	281	1.54	3	337
4	1	3.3	144	4.9	114	2.58	0.8	1.15	5.4	3.18	82	1	143	31	18	70	28	42	0.67	208	1.59	2	203
4	3	2.9	144	5.5	115	2.62	0.82	1.25	4.4	3.02	71	1	182	28	21	67	28	39	0.72	628	1.28	2	219
4	4	2.2	148	5.7	113	2.49	0.77	1.33	4.4	2.85	71	2	190	25	14	62	26	36	0.72	106	0.83	4	205
5	1	2	136	4.7		2.66	0.81	1.58	16.4	21.2	63		160				43			19	14.5		
5	2									2.75													
5	3	4.2	144	5.3	114	2.41	0.89	1.36		4.42	59	1	134	18	27	69	43	26	1.65	120	1.39	4	
5	4	2.1	142	4.8	113	2.23	0.75	1.22	6.6	6.46	59	0	159	13	22	72	31	41	0.76	126	6.13	5	232
6	1	4.7	144	4.6	110	2.68	0.72	1.28	6.8	10.6	136	0	91	19	27	63	29	34	0.85	92	1.54	5	296
6	2	2.6	148	4.3	111	2.61	0.55	1.04	6	5.67	98	0	146	20	36	57	29	28	1.04	117	1.28	3	280
6	3	2.6	148	4.6	115	2.62	0.65	1.74	5.5	5.81	99	1	177	15	32	58	28	30	0.93	94	2.51	5	221
6	4	2.1	151	3.9	113	2.52	0.74	1.74	5.1	4.44	100	2	232	15	21	60	31	29	1.07	35	1.01	4	209
7	1	6.7	141	4.2	108	2.73	0.76	1.48	5.6	33.1	88	1	60	17	76	78	37	41	0.90	85	5.13	12	324
7	2	8.1	143	5.4	112	2.65	0.72	1.4	4.8	9.69	81	0	88	25	324	68	39	29	1.34	184	1.23	25	303
7	3	5.5	143	4	111	2.53	0.68	1.38	5.4	8.3	78	0	75	22	379	64	37	27	1.37	125	0.84	15	280
8	1	6.1	143	4.6	111	2.89	0.83	1.44	5.4	17.4	110	1	53	28	36	69	33	36	0.92	161	5.35	4	400
8	3	5.8	146	4.4	115	2.76	0.73	1.26	6.5	6.55	116	1	94	22	42	62	34	28	1.21	110	1.3	4	283
8	4	1.9	151	4.1	114	2.62	0.72	1.55	5.1	4.99	98	0	210	15	18	61	28	33	0.85	47	1.3	5	213

Appendix 34 (continued)

Dog	Visit	urea	Na	K	Cl	Ca	Mg	PO4	Glu	Chol	Crea	TBil	ALKP	AST	ALT	T P	Alb	Glob	A:G	CK	Trig	GGt	Fruct
9	1	10.1	145	5	104	3	1.02	1.26	6	4.99	105	2	2044	60	165	80	46	34	1.35	65	0.65	3	287
9	2	7.4	145	4.2	109	3.24	0.51	1.37	5.9	3.6	69	0	1702	67	204	71	40	31	1.29	91	0.66	3	269
9	3	5.8	148	4.3	103	3.16	0.83	1.2	6.7	5.57	76	2	1244	29	143	65	42	23	1.83	72	0.78	3	220
9	4	4.9	145	3.6	110	3.05	0.69	0.6	5.8	2.78	96	2	831	40	154	69	36	33	1.09	105	0.34	4	156
10	1	3.5	144	4.5	116	2.59	0.89	1.05	5	9.96	104	1	82	17	23	62	35	27	1.30	102	1.49	5	303
10	2	3.1	146	4.1	117	2.59	0.81	1.04	5.4	7.2	101	1	73	20	22	61	33	28	1.18	198	1.42	4	295
10	3	3.1	145	4.3	110	2.53	0.71	1.21	5.4	7.07	93	2	116	16	28	61	31	30	1.03	161	1.45	8	262
10	4	2.8	147	3.9	115	2.55	0.76	0.98	5.8	6.87	95	0	115	17	23	63	34	29	1.17	90	1.72	6	276
11	1	4	143	4.5	115	2.79	0.7	1.26	5.2	7.4	127	1	61	21	31	66	37	29	1.28	147	0.86	1	271
11	2	5	141	4.4	121	2.63	0.91	1.17	5.3	6.63	121	0	102	27	42	64	34	30	1.13	248	1.3	3	257
11	3	3.6	147	5.3	115	2.99	0.77	1.11	5.3	7.02	117	1	107	19	47	67	36	31	1.16	333	0.9	3	245
11	4	4.9	147	4.1	111	2.87	0.73	1.31	5.8	4.95	106	0	119	18	49	61	32	29	1.10	104	0.71	4	203
12	1	5.4	144	4.7	112	3.04	0.72	1.27	5.5	22.3	150	1	82	24	33	72	38	34	1.12	100	11.1	4	353
12	2	7	146	4.4	115	3.07	0.88	1.58	5.4	6.3	138	0	65	23	31	71	42	29	1.45	108	1.07	2	362
12	3	6.9	148	4.4	111	2.76	0.77	1.5	4.8	4.61	142	0	50	20	46	61	35	26	1.35	87	1.08	4	357
13	1	9.7	139	4.7	114	2.37	0.89	1.04	5	10.3	152	1	53	27	32	61	29	32	0.91	99	1.05	3	264
13	2	7.7	146	4.2	116	2.38	1.03	1.16	5	4.87	127	2	64	22	38	62	31	31	1.00	60	0.48	3	268
13	3	6.9	147	4.3	116	2.61	0.92	1.25	4.2	5.19	113	1	76	24	38	59	30	29	1.03	79	0.49	5	249
13	4	7.2	149	4.8	113	2.68	1.04	1.2	4.4	6.03	136	1	57	22	28	62	32	30	1.07		0.73	4	
14	1	4	144	4.1	109	2.68	0.87	1	5.6	13.6	98	2	47	16	29	59	34	25	1.36	47	1.39	6	372
14	2	5.1	147	4.1	119	2.55	0.8	1.45	4.5	6.77	94	0	46	14	32	58	37	21	1.76	67	0.65	6	304
14	3	4.3	145	4.4	115	2.61	0.71	1.68	5	8.6	80	1	70	15	34	58	33	25	1.32	83	0.67	3	317
14	4	4.9	144	4.1	117	2.37		1.43	5.5	8.5	90	2	79	14	25	50	28	22	1.30	54		6	255
15	1	4.3	144	4.4	111	2.86	0.71	1.13	5.1	13.2	130	2	122	18	18	67	38	29	1.31	52	0.86	5	319
15	2									3.79													308
15	4	4.5	147	4.2	114	2.76	0.65	1.43	4.9	5.03	100	1	101	20	21	57	37	20	1.85	57	0.48	4	298
16	1	8.8	147	4.6	99	2.39	0.66	1.48	5.3	7.05	106	1	89	112	154	56	31	25	1.24	1039	1.37	4	327
16	2	6.9	147	4.5	111	2.73	0.65	1.29	5.2	5.02	105	2	254	29	266	58	34	24	1.42	78	0.68	10	300
16	3	4	146	4.6	114	2.51	0.46	1.55	6.4	3.96	100	1	83	27	74	57	31	26	1.19	149	1.01	3	300
16	4	4.1	144	4.5	112	2.5	0.5	1.3	6	4.63	96	1	99	25	51	58	32	26	1.23	146	0.94	2	255
17	1	4.7	144	4.2	111	2.84	0.95	1.5	5.6	9.37	91	0	137	26	54	88	53	35	1.51	101	7.95	3	345
17	2	5.1	148	4	118	2.93	0.96	1.44	6	5.63	75	0	104	32	26	78	51	27	1.89	311	1.6	3	338
17	3	6.4	148	3.7	127	2.52	0.88	1.19	5.6	5.86	73	2	61	6	21	89	48	41	1.17	131	4.27	5	372

Appendix 34 (continued)

Dog	Visit	urea	Na	K	Cl	Ca	Mg	PO4	Glu	Chol	Crea	TBil	ALKP	AST	ALT	T P	Alb	Glob	A:G	CK	Trig	GGt	Fruct
17	4	4.8	147	4.9	112	2.7	0.89	1.22	6	6.87	73	0	98	19	20	77	50	27	1.85	437	2.17	3	298
18	1	2.2	146	4.3	103	2.65	0.87	1.22	7.8	2.15	93	3	809	36	338	66	38	28	1.36	94			233
18	3	2.1	146	4.2	114	2.42	0.5	1.41	5.6	3	69	3	318	18	165	61	35	26	1.35	60	0.5	13	250
18	4	3.4	146	4.7	112	2.49	0.58	1.44	5.6	3.31	85	1	95	16	22	57	32	25	1.28	78	0.65	7	252
19	1	8.5	144	4.8	104	2.77	0.75	1.56	5.3	10.2	133	1	126	32	47	63	33	30	1.10	191	0.85	13	292
19	2																						
20	1	4.4	131	5.4	95	2.44	0.78	1.31		17.9	118	1	1550	16	73	62	29	33	0.88	90	1.03	7	705
20	3																						
21	1	4.8	146	4.9	111	2.52	0.82	1.41	5.2	11.8	95	1	392	34	269	65	36	29	1.24	90	0.48	10	270
21	2																						246
21	4	5.1	146	4.4	118	2.24	0.79	1.22	5.2	4.39	110	1	65	21	32	61	36	25	1.44	97	0.73	4	251
22	1	4.7	145	4.2	108	2.79	0.99	1.2	5.4	12.5	98	1	138	65	597	68	42	26	1.62	286	1.11	22	338
22	2	5.3	148	4	127	2.73	0.74	1.37	6.1	7.19	74	0	79	34	220	78	43	35	1.23	87	2.83	5	296
22	3	3.9	147	3.8	111	2.63	0.71	1.62	5.7	4.51	70	3	80	27	89	64	39	25	1.56	132	0.49	4	264
22	4	3.8	148	4.1	125	2.67	0.71	1.32	6.4	4.51	85	0	69	29	43	71	41	30	1.37	123	2.15	1	110
23	1	6	145	5	114	2.61	0.77	1.25	5.4	19.6	155	2	371	34	87	59	31	28	1.11	100	4.42	4	305
23	2	4.2	148	4.5	113	2.56	0.69	1.29	4.7	6.55	102	3	220	42	103	55	33	22	1.50	110	1.13	2	298
23	3	4.3	148	4.1	112	2.49	0.64	1.46	5	4.01	95	1	113	21	32	53	32	21	1.52	100	0.69	2	223
23	4	5	146	4	114	2.41	0.72	1.61	5.1	4.73	105	0	101	19	24	52	33	19	1.74	123	0.89	4	221
24	1	6.6	147	4.8	115	2.54	0.85	1.41	5.8	4.04	139	1	464	21	85	55	25	30	0.83	120	0.58	4	226
24	2	6	146	4.8	116	2.45	0.75	1.33	5.9	3.86	146	1	466	23	79	53	26	27	0.96	142	0.63	3	236
24	3	8	148	4.9	114	2.4	0.94	1.71	5.5	3.65	109	1	488	21	76	49	24	25	0.96	206	0.6	5	208
24	4	11	148	4.9	114	2.47	0.91	1.68	5.4	4.52	121	2	495	19	126	52	25	27	0.93	72		4	219
25	1	8.8	142	5.3	101	2.73	0.9	1.82	5.4	13.5	94	0	314	19	25	73	40	33	1.21				
25	2																						
26	1	4.2	146	4.2	115	2.67	0.71	0.98	5.1	10.9	103	1	181	26	53	68	34	34	1.00	82	3.51	3	259
26	2	4.9	150	6.2	112	2.12	0.59	1.92		7.76	77	0	254	39	162	65	34	31	1	200	2.51	5	235
27	1	5.4	147	4.2	112	2.83	0.67	1.15	10.1	10.5	124	1	174	20	146	63	34	29	1.17	106	3.35	5	359
27	2	4.7	147	4.2	111	2.81	0.59	1.63	5.3	4.54	93	2	195	22	203	64	38	26	1.46	107	1.26	5	360
27	3	3.3	146	4.6	113	2.78	0.83	1.56	6	3.45	93	0	103	24	123	64	35	29	1.21	91	0.9	4	307
27	4	5.5	148	4.5	117	2.56	0.94	1.43	5.5	3.28	92	0	106	15	60	64	35	29	1.21	120	1.36	4	323
28	1	2.9	144	4.5	114	2.53	0.72	1.08		7.11	105	2	332	65	504	57	35	22	1.59				275
28	2																						

Appendix 34 (continued)

Dog	Visit	urea	Na	K	Cl	Ca	Mg	PO4	Glu	Chol	Crea	TBil	ALKP	AST	ALT	T P	Alb	Glob	A:G	CK	Trig	GGt	Fruct
28	3																						250
29	1	2.9	146	5.8	110	2.67	0.9	1.44		9.5	68	1	501	14	31	69	34	35	0.97	45	1.96	5	423
29	2								4														382
29	4	2.6	146	3.7		2.46	0.69	1.36	6.2	6.82	73	1	178	8	20	85	38	47	0.81	50	5.96	6	310
30	1	7.6	144	4.7	107	2.68	0.91	1.27	5.5	16.2	141	2	24	35	56	70	39	31	1.26	202	5.41	4	321
30	2	8.6	144	5.8	106	2.82	0.9	1.85		5.36	124	1	36	31	56	65	39	26	1.50	139	0.6	3	340
30	3	7.1	146	5.2	120	2.76	0.8	1.83	6.1	3.82	140	0	14	32	43	72	43	29	1.48	133	2.67	3	310
31	1	1.7	140	3.6	98	2.44	0.62	1.23	8.1	12.5	65	0	128	25	40	64	33	31	1.06	182	1.69	6	293
31	2	1.6	149	4.8	106	2.55	0.68	1.48	5.7	6.23	58	0	126	17	52	60	35	25	1.40	172		14	225
31	3	3.5	149	4.2	133	2.72	0.75	1.23	8.1	6.86	64	5	94	32	22	91	37	54	0.69	114	5.32	7	247

For explanation of biochemical abbreviations and relevant measurement units see Appendices 3 and 4. Dog and visit numbers correspond to those in Appendix 33.

Appendix 35

Haematological data recorded from 31 dogs receiving thyroid hormone replacement therapy.

Dog	Visit	RBC	Hb	HCT	MCV	MCH	MCHC	PLT	MPV	WBC	Band N	Neut	Lymp	Mono	Eosin
1	1	4.89	11.5	33.4	68	23.5	34.4	398	8.7	10.7	0.214	6.955	2.033	0.963	0.535
1	3														
1	4	4.96	11.3	32.9	66	22.7	34.3	198	9.9	8	0	4.56	2.4	0.48	0.56
2	1	5.25	12.2	35	67	23.2	34.8	340	8.2	9.1	0	6.734	2.093	0.273	0
2	2	5.6	13.2	37.6	67	23.5	35.1	315	8.4	15.2	0.456	10.184	2.736	1.824	0
2	3	5.9	14	41.3	70	23.7	33.8	243	8.7	16.1	0	13.363	1.932	0.805	0
2	4	6.58	15.5	45.9	70	23.5	33.7	244		14.3	0.501	11.3	1.716	0.715	0.072
3	1	6.48		41.2	64			780	8.3	9.7	0.097	6.79	2.231	0.485	0.097
3	2	6.18	13.2	39.2	63	21.3	33.6	513	9.9	14.3	0	7.722	5.434	0.858	0.286
3	3	6.15	12.7	37.2	60	20.6	34.1	761	9.4	13.8	0	9.798	3.036	0.552	0.414
3	4	7.09	13.6	39.2	55	19.1	34.6	730	9.3	10.4	0	6.552	2.912	0.728	0.208
4	1	5.6	14	41.9	75	25	33.4	143	11.5	11	0.44	5.83	1.65	1.43	0.33
4	3	5.59	14.3	43.5	78	25.5	32.8	123	10.2	6.5	0	4.16	1.43	0.195	0.325
4	4	5.37	12.7	39.1	73	23.6	32.4	77	10.4	9.2	0.184	5.52	2.3	0.644	0
5	1	5.52		34.7	63			353	8.9	11.4	0	8.89	1.59	0.57	0.34
5	2														
5	3														
5	4	6.89	15.1	43.5	63	21.9	34.7	177	10.6	12.1	0.242	9.317	0.847	1.089	0.363
6	1	6.32	14.3	41.4	66	22.6	34.5	286	9.6	11	0	7.92	2.31	0.33	0.44
6	2	5.93	13.9	41.1	69	23.4	33.8	277	9.9	17	0	14.62	1.36	0.17	0.51
6	3	7.33	17.5	49.1	67	23.8	35.6	280	9.3	17.6	0.352	14.608	1.76	0.352	0.528
6	4	9.03	19.1	54.4	60	21.1	35.1	209	9.3	16.4	0	13.448	2.296	0.328	0.328
7	1	5.06	12	34	67	23.7	35.2	530	7	5.7	0	3.591	1.824	0.228	0.057
7	2	5.38	12.9	36.7	68	23.9	35.1	565	6.9	8.7	0	6.786	1.044	0.696	0.087
7	3	6.41	15	43.1	67	23.4	34.8	456	7.3	8.5	0	6.545	1.53	0.255	0.255
8	1	4.96	11.9	34.3	69	23.9	34.6	245	8.4	11.8	0.23	9.08	1.29	0.59	0.47
8	3	5.84	14.2	38.7	66	24.3	36.6	315	8	17.4	0.522	13.05	2.436	0.696	0.696
8	4	6.73	14.7	40.8	61	21.8	36	317	8.3	19.5	0.195	14.43	2.925	0.585	1.365

Appendix 35 (continued)

Dog	Visit	RBC	Hb	HCT	MCV	MCH	MCHC	PLT	MPV	WBC	Band N	Neut	Lymp	Mono	Eosin
9	1	5.17	11.7	33.9	66	22.6	34.5	783	9	15.4	0	13.09	1.232	0.616	0.308
9	2	5.5	12.4	36.9	67	22.5	33.6	417	10.5	13.2	0	9.9	1.452	0.528	0
9	3	5.88	13.7	44.2	75	23.2	30.9	142	9.5	16.2	0	13.122	2.268	0.324	0.324
9	4	7.06	15.4	43.5	62	21.8	35.4	433	9.6	10.1	0.202	7.878	1.212	0.606	0.202
10	1	6.51	16.3	45.2	69	25	36	212	9.1	6.7	0	3.484	2.948	0.201	0.067
10	2	6.52	16.1	46.5	71	24.6	34.6	229	9	8.3	0	4.067	3.403	0.332	0.415
10	3	6.62	15.4	44.8	68	23.2	34.3	244	8.8	8.3	0	4.233	3.735	0.083	0.249
10	4	7.22	16.8	48.6	67	23.2	34.5	213	9.2	10.8	0	7.884	2.484	0.216	0.216
11	1	6.55	15.2	41.8	64	23.2	36.3	290	7.9	6.2	0	4.526	1.302	0.248	0.124
11	2	6.43	15	48.1	75	23.3	31.1	214	9.8	8.1	0.405	5.994	1.134	0.162	0.324
11	3	6.75	15	41.8	62	22.2	35.8	322	8.5	7.1	0	5.68	0.639	0.497	0.213
11	4	6.55	14.4	41.8	64	21.9	34.4	510	8.3	11.1	0	8.88	1.332	0.333	0.555
12	1														
12	2	5.47	13	37.6	69	23.7	34.5	347	8.6	12.6	0.252	8.694	2.142	0.756	0.252
12	3	6.3	13.7	41.1	65	21.7	33.3	384	8.2	13.1	0.131	9.694	1.572	0.655	1.048
13	1	6.63	15.9	43.7	66	23.9	36.3	184	9.4	4.7	0	2.303	1.457	0	0.94
13	2	7.41	18.1	49.9	67	24.4	36.2	243	9	6.4	0	3.776	1.216	0.064	1.344
13	3	7.91	19.1	53.4	68	24.1	35.7	244	8.8	8	0.08	5.44	1.28	0.24	0.96
13	4	8.25	19.5	54.4	66	23.6	35.8	133	10	7.4	0	5.032	1.702	0.074	0.592
14	1	5.89	13.5	37.4	63	22.9	36	404	7.5	8.5	0	6.375	1.445	0.595	0.085
14	2	6.12	14.3	39.4	64	23.3	36.2	416	7.9	8.2	0	5.576	2.132	0.41	0.082
14	3	6.41	14.5	40.6	63	22.6	35.7	476	8	10.1	0	6.767	3.232	0.101	0
14	4	6.55	14.3	42.1	64	21.8	33.9	401		11.2		7.84	2.912	0.28	0.056
15	1	6.27	14.8	43.7	70	23.6	33.8	213	9.3	6.4	0	4.16	2.11	0.06	0.06
15	2														
15	4	6.71	16.2	45.2	67	24.1	35.8	219	9	10.7	0.107	6.634	2.889	0.642	0.428
16	1	4.49	11.3	32	71	25.1	35.3	394	7.3	12.7	0	9.144	2.54	0.889	0.127
16	2	5.2	12.9	37.4	72	24.8	34.4	446	7.5	13.4	0	9.782	2.948	0.134	0.268
16	3	6.03	14.2	41.5	69	23.5	34.2	422	7.7	18.4	0.552	13.8	3.312	0.184	0.552
16	4	6.09	14.3	40.5	67	23.4	35.3	362	8	13.6	0	10.336	2.176	0.816	0.272
17	1	5.72	14.1	41.2	72	24.6	34.2	719	8.3	9.5	0	7.22	1.71	0.57	0
17	2	6.82	16.9	46.2	68	24.7	36.5	624	9.2	15.3	0	12.7	1.99	0.61	0
17	3	7.1		46.8	66			463	9.1	12.7	0	10.287	1.905	0.508	0

Appendix 35 (continued)

Dog	Visit	RBC	Hb	HCT	MCV	MCH	MCHC	PLT	MPV	WBC	Band N	Neut	Lymp	Mono	Eosin
17	4	7.37	17.2	47.6	65	23.3	36.1	386	8.7	12.8	0	10.368	1.92	0.512	0
18	1	5.08	11	33.3	66	21.6	33	190	9.6	21.2	0.212	18.868	0.636	0.848	0.636
18	3	5.59	11.5	35.6	64	20.5	32.3	162	10.4	12	0.12	8.16	1.8	1.2	0.6
18	4	6.51	12.9	40.2	62	19.8	32	187	10	9.6	0.288	7.008	1.824	0.384	0.096
19	1	5.63	12.3	36.3	64	21.8	33.8	465	7.5	7.7	0.077	5.005	1.386	0.77	0.462
19	2														
20	1	5.57	12.9	37.8	68	23.1	34.1	486	8.1	10.3	0	7.622	1.854	0.618	0.103
20	3														
21	1	6.24	13.9	41.8	67	22.2	33.2	327	7.9	5.6	0	2.91	2.24	0.39	0.05
21	2														
21	4	7.13	16.3	45.2	63	22.8	36	260	8	7	0	5.04	1.4	0.42	0.14
22	1	6.18	13.9	39.4	64	22.4	35.2	365	8.1	8.8	0	5.368	1.584	0.704	1.144
22	2	5.54	13.6	35.3	64	24.5	38.5	515	7.6	10	0	7.1	1.6	0.3	1
22	3	6.28	14.4	39.7	63	22.9	36.2	338	8	14.3	0.143	11.011	1.716	0.858	0.429
22	4	6.67	15	41.1	62	22.4	36.4	408	8.4	15.8	0.158	12.956	1.58	0.632	0.474
23	1	5.41	12.9	36.6	68	23.8	35.2	356	6.8	7	0	3.36	2.52	0.14	0.91
23	2														
23	3	6.86	15.1	43.1	63	22	35	412	7.5	9.9	0	5.94	2.772	0.693	0.495
23	4	7.06	16	43.5	62	22.6	36.7	292	7.6	9.2	0	5.888	2.484	0.46	0.368
24	1	4.86	12.1	34.1	70	24.7	35.4	513	6.9	7.8	0	6.006	1.092	0.312	0.39
24	2	4.59	11.2	32.3	70	24.4	34.6	486	7.3	7.5	0	6.45	0.825	0.15	0.075
24	3	5.53	13	38.7	70	23.5	33.5	351	7.5	7.6	0	4.712	1.596	0.38	0.912
24	4	5.84	14.3	41.1	70	24.4	34.7	398	7.4	7.2	0.36	4.824	1.08	0.216	0.72
25	1	4.79	10.7	31	65	22.3	34.5	682	8.3	10.5	0	7.56	1.365	1.05	0.315
25	2														
26	1	6.73	14.3	41.8	62	21.2	34.2	348	9.3	7.2	0	5.184	1.8	0.072	0.144
26	2	6.41	14.2	41.8	65	22.1	33.9	342	9.6	12.3	0.615	9.594	0.861	0.861	0.123
27	1	4.61	11.2	30.6	66	24.2	36.6	381	8.1	5.2	0	1.768	2.496	0.572	0.364
27	2	5.11	12.2	34.6	68	23.8	35.2	338	8.1	10.8	0	8.1	1.296	0.864	0.54
27	3	5.81	13.3	38.6	66	22.8	34.4	271	9.3	12.1	0	8.712	2.178	0.605	0.605
27	4	7.28	15.1	43.5	60	20.7	34.7	258	8.9	9.1	0	5.824	2.366	0.364	0.546
28	1														
28	2														

Appendix 35 (continued)

Dog	Visit	RBC	Hb	HCT	MCV	MCH	MCHC	PLT	MPV	WBC	Band N	Neut	Lymp	Mono	Eosin
28	3														
29	1	5.46		34.7	64			420	10.5	13.6	0.272	9.792	2.176	0.952	0.408
29	2														
29	4	6.19	15.1	39.1	63	24.3	38.6	460	8.6	16.9	0.338	11.154	3.887	0.338	1.014
30	1	5.01	14.9	39.6	71	26	35.3	170	9.2	11.9	0	8	2.01	0.35	0.34
30	2	5.99	15.5	43.8	73	25.8	35.3	174	9.2	11.2	0	7.16	2.35	0.44	0.44
30	3	6.86	16.8	49.9	73	24.4	33.6	232	9.3	12.5	0	9.37	2.75	0	0.25
31	1	5.94	12.9	38.6	65	21.7	33.4	479	9	38.1	1.524	33.528	1.524	1.143	0.381
31	2	6.24	13.4	39.2	63	21.4	34.1	472	8.4	10.5	0	8.085	1.89	0.21	0.21
31	3	7.69	16.9	45.6	59	21.9	37	582	9	10.7	0	8.025	2.247	0.214	0.214

For explanation of haematological abbreviations and relevant measurement units see Appendices 3 and 4. Dog and visit numbers correspond to those in Appendix 33.

Appendix 36

Detail of 205 cases with various disorders, including signalment, drug therapy, endocrine results and case outcome data.

Case Number	Age (yrs)	Breed	Sex	Category	Method	Disease course	CDS	Suppressive therapy ?	Any drug therapy ?	Thyroid Therapy ?	total T_4	cTSH	free T_4	total T_3	TgAb	Outcome
136261	11.00	Cocker spaniel	M	General Medicine	PM	C	8	YES	YES	NO	7.89			1.01	E	E
135675	6.00	German shepherd	M	Other	CP	A	8	NO	YES	NO	33.33	0.19	32.33	1.48	E	F
134768	2.58	Saluki	MN	Gastrointestinal	HP	C	4	YES	YES	NO					N	P
136270	0.67	Akita	M	Orthopaedic	IMG	C	3	YES	YES	NO					N	F
132633	8.25	Retriever	F	Orthopaedic	CP	C	6	YES	YES	NO	44.40	0.13		1.59	N	P
136301	14.00	Collie cross	M	Neoplasia	HP	C	7	NO	YES	NO	24.32	0.04	32.04	0.79	N	E
136190	10.00	Labrador retriever	M	Gastrointestinal	HP	C	5	NO	YES	NO	9.07	0.42	11.78	1.01	N	E
136290	10.00	Crossbreed	M	Soft Tissue	Clin	C	2	NO	NO	NO	17.58	0.35		1.37	N	P
136153	1.00	Crossbreed	MN	Trauma	Clin	A	3	YES	YES	NO	26.06	0.04	24.77	1.69	N	F
136291	2.00	Border terrier	M	Gastrointestinal	Clin	C	2	NO	YES	NO	13.82	0.08		1.55	N	F
136125	2.00	Spinone	F	IM	CP	C	5	YES	YES	NO	4.70	0.01	5.29	0.85	N	P
133668	8.00	Hungarian viszla	M	Neoplasia	IMG	C	2	YES	YES	YES	10.29	0.22	13.11	2.81	N	E
136297	1.42	Border collie	FN	Other	CP	A	6	YES	YES	NO	4.70	0.03		0.59	N	F
135644	11.00	Boxer	FN	Neoplasia	HP	C	1	NO	YES	NO	8.47	0.72	11.95	1.18	N	P
136314	2.50	Golden retriever	F	Trauma	Clin	C	5	NO	YES	NO	6.63	0.03	26.59	0.59	N	F
136311	10.00	SBT	M	Neoplasia	IMG	C	2	NO	NO	NO	14.80	0.12	15.91	1.90	E	P
132480	8.08	English Springer Spaniel	M	Neoplasia	HP	C	2	NO	NO	NO	26.21	0.08	25.43	2.03	N	F
136313	5.00	Irish Wolfhound	M	Dermatological	HP	C	2	YES	YES	NO	8.74	0.07	23.60	1.34	N	P
136310	10.00	Labrador	M	Neoplasia	HP	C	1	NO	NO	NO	8.05	0.28	11.50	1.20	N	F
136341	5.00	WHFT	FN	Gastrointestinal	CP	C	2	NO	YES	NO	15.53			1.03	N	F
136340	12.00	Miniature Schnauzer	M	Neoplasia	IMG	C	6	NO	YES	NO	32.26	0.36		1.93	N	E
132521	2.00	St Bernard	F	Cardiovascular	IMG	C	3	NO	NO	NO	20.35	0.66	21.55	1.05	N	F
136280	12.00	Retriever	FN	Neoplasia	IMG	C	8	NO	NO	NO	20.75	0.18	16.11	1.43	N	E
136353	0.67	Retriever	M	Gastrointestinal	IMG	C	3	NO	YES	NO	28.37	0.06	24.40	1.12	N	F
136348	9.42	Rough collie	FN	Orthopaedic	IMG	C	3	NO	NO	NO	26.56	1.08	25.41	1.72	N	D
135627	8.00	Retriever cross	MN	Dermatological	HP	C	3	YES	YES	NO	7.49	0.33	16.04	1.16	N	P

Appendix 36 (continued)

Case Number	Age (yrs)	Breed	Sex	Category	Method	Disease course	CDS	Suppressive therapy ?	Any drug therapy ?	Thyroid Therapy ?	total T_4	cTSH	free T_4	total T_3	TgAb	Outcome
133631	10.75	Retriever	F	Endocrine	CP	C	3	YES	YES	NO	23.97	0.14		1.11	N	P
135000	14.00	Retriever	MN	Respiratory	IMG	C	7	NO	NO	NO	21.66	0.41	17.80	3.20	N	P
136364	4.00	JRT	M	Ophthalmological	Clin	A	3	NO	NO	NO	18.02	0.09	15.51	1.87	N	F
136374	13.00	SBT	M	Cardiovascular	Other	C	6	NO	YES	NO	12.43	0.49	15.55	1.35	N	P
129441	10.25	Crossbreed	M	Urogenital	CP	C	4	YES	YES	NO	6.56	0.39	9.44	0.98	N	E
134175	10.25	Retriever	FN	Neoplasia	HP	C	5	YES	YES	NO	4.70	0.04	10.53	1.43	N	P
136388	2.00	Maltese terrier	M	General Medicine	CP	C	2	NO	NO	NO	20.40	0.12	20.52	1.48	N	F
136387	2.92	Terrier	M	Neoplasia	PM	A	6	YES	YES	NO	4.70	0.04	12.48	0.72	N	E
136389	0.75	Setter	F	Orthopaedic	Clin	C	1	NO	NO	NO	28.88	0.09	34.13	1.52	N	F
136391	5.00	Dachshund	M	Neoplasia	IMG	C	3	NO	YES	NO	10.31	0.32		0.95	N	E
136127	1.00	Lurcher	MN	Gastrointestinal	CP	C	2	NO	YES	NO	10.74	0.05	19.98	1.44	N	F
136386	13.00	Dachshund	MN	General Medicine	Clin	C	2	NO	YES	NO	38.82	0.03		1.80	N	F
136404	5.50	St Bernard	FN	Neoplasia	HP	C	3	NO	YES	NO	20.41	0.17	20.31	0.96	N	E
136379	5.00	Bull terrier	F	General Medicine	IMG	C	2	NO	YES	NO	17.01	0.29	25.62	1.20	N	P
132699	11.42	Cocker spaniel	M	Gastrointestinal	CP	C	2	NO	YES	NO	18.64	0.22	18.30	2.82	N	P
136406	3.00	Retriever	F	Respiratory	CP	C	5	NO	YES	NO	9.55	0.04	32.87	0.14	N	P
136417	8.00	Crossbreed	FN	Neurological	Clin	A	8	NO	YES	NO	22.18	0.05	28.35	1.06	N	E
136415	4.00	Irish wolfhound	M	Neoplasia	HP	A	3	YES	YES	NO	20.65	0.05	24.73	1.23	N	E
136420	0.33	Great Dane	M	Other	Clin	A	2	YES	YES	NO	32.61	0.25	38.11	1.40	N	F
136419	11.42	GSD	MN	Orthopaedic	CP	C	6	YES	YES	NO	24.88	0.37	17.08	1.06	N	F
136421	0.92	Bullmastiff	F	Gastrointestinal	CP	C	2	NO	YES	NO	28.77	0.08	31.77	1.45	N	F
127966	15.00	Border collie	FN	Orthopaedic	IMG	C	5	YES	YES	NO	19.97	0.77	19.87	1.94	N	P
136423	1.83	Crossbreed	M	Other	Clin	A	2	NO	NO	NO	29.34	0.06	34.13	1.47	N	F
133626	12.00	Retriever	FN	Neoplasia	HP	C	6	NO	YES	NO	4.70	0.17	9.21	0.87	N	P
133356	11.75	Crossbreed	FN	Endocrine	CP	C	2	NO	YES	NO	19.74	0.02		1.76	N	P
131733	11.83	GSP	M	Orthopaedic	IMG	A	6	NO	NO	NO	23.61	0.14	53.11	1.36	N	E
136429	11.00	GSD	M	Ophthalmological	Clin	C	3	NO	YES	NO	26.42	0.44	20.43	1.92	N	F
136426	3.33	Rottweiler	M	Gastrointestinal	CP	C	2	YES	YES	NO	13.06	0.19	16.44	0.74	E	P
125014	5.25	Beagle	FN	General Medicine	CP	C	2	YES	YES	NO	22.31	0.03		1.84	N	F
136438	5.00	Bull Mastiff	M	Neoplasia	HP	A	8	NO	NO	NO	7.87	0.02		0.44	N	E
136451	8.00	Crossbreed	M	Neoplasia	HP	C	2	NO	YES	NO	18.28	0.14	22.77	1.95	N	F
136446	0.58	Bull Mastiff	M	Cardiovascular	IMG	C	4	NO	YES	NO	48.71	0.03	22.46	1.77	N	P
136254	7.08	Border collie	MN	Orthopaedic	CP	A	3	YES	YES	NO	20.19	0.44		1.65	N	F

Appendix 36 (continued)

Case Number	Age (yrs)	Breed	Sex	Category	Method	Disease course	CDS	Suppressive therapy ?	Any drug therapy ?	Thyroid Therapy ?	total T_4	cTSH	free T_4	total T_3	TgAb	Outcome
136466	12.75	Terrier	M	General Medicine	CP	C	7	YES	YES	NO	8.04	0.42	10.44	1.10	N	E
119382	11.00	Cocker spaniel	FN	Neoplasia	IMG	C	9	NO	YES	NO	5.28	0.51	19.97	0.63	N	E
136476	1.50	Cocker spaniel	M	Cardiovascular	IMG	C	7	NO	NO	NO	13.49	0.02	25.26	0.82	N	P
136229	6.00	Pointer	FN	Trauma	Clin	C	1	NO	YES	NO	37.73	0.07	38.96	2.14	N	F
131494	9.00	CKCS	M	Respiratory	IMG	C	7	YES	YES	NO	16.24	0.16	21.03	3.05	N	P
135935	4.00	Retriever	FN	Neoplasia	HP	C	6	YES	YES	NO	30.73	0.12	20.61	5.64	N	P
136498	12.50	Irish Setter	M	Respiratory	Clin	C	4	NO	NO	NO	15.30	0.05	15.15	2.07	N	F
136512	6.00	Yorkshire Terrier	M	Neurological	Clin	C	4	NO	NO	NO	13.03	0.26	9.40	1.33	N	F
136491	1.33	Lurcher cross	FN	General Medicine	CP	C	2	NO	YES	NO	25.04	0.05	25.22	1.37	N	F
136525	8.00	GSD	M	Gastrointestinal	Clin	C	2	YES	YES	NO	20.24	0.12	18.08	1.33	N	F
136526	9.00	English springer	MN	Cardiovascular	IMG	C	5	NO	YES	NO	32.85	0.18	36.50	1.42	N	P
135869	2.25	Golden retriever	MN	Gastrointestinal	HP	C	2	YES	YES	NO	24.18	0.02	25.25	1.06	N	F
122289	9.67	Retriever cross	FN	Orthopaedic	IMG	A	2	YES	YES	NO	24.96	0.19	15.83	1.35	N	F
136533	1.50	Border collie	F	Gastrointestinal	CP	A	8	NO	NO	NO	13.31	0.02	14.22	1.29	N	E
134621	3.75	Bernese mountain dog	M	Neoplasia	CP	C	7	YES	YES	NO	11.26	0.05	7.04	0.96	N	P
136507	4.50	Basset Hound	M	Neoplasia	HP	A	1	YES	YES	NO	18.09	0.27	12.31	1.12	N	E
136554	13.00	WHWT	FN	Cardiovascular	IMG	C	3	NO	YES	NO	24.70	0.21	14.58	1.09	N	P
136561	12.50	Collie cross	FN	Orthopaedic	IMG	C	3	NO	NO	NO	12.74	0.10	14.71	1.18	N	P
134873	5.67	Tibetan spaniel	MN	Immune Mediated	CP	A	3	YES	YES	NO	29.06	0.41	19.16	1.60	N	P
136536	5.00	Labrador retriever	F	Neoplasia	IMG	C	4	NO	YES	NO	6.96	0.09	22.49	0.58	N	E
136534	6.75	Cocker spaniel	MN	Neoplasia	HP	C	10	YES	YES	NO	6.69	0.13	23.31	0.34	N	E
136539	1.08	GSD	F	Gastrointestinal	Clin	C	3	YES	YES	NO	27.16	0.06	29.46	1.01	N	F
136548	5.00	Flat coated retriever	M	Neoplasia	CP	C	1	NO	YES	NO	27.28	0.09	6.72	1.02	N	F
135489	1.00	Samoyed	F	Orthopaedic	IMG	C	1	YES	YES	NO	9.74			0.91	E	F
135224	2.50	Golden retriever	M	Orthopaedic	IMG	C	1	NO	NO	NO	30.41	0.03		2.02	N	F
136568	8.00	Cairn terrier	FN	Endocrine	CP	C	4	YES	YES	YES	30.46	0.14	35.19	10.34	N	P
136566	8.50	Labrador	FN	General Medicine	CP	C	8	NO	NO	NO	10.42	0.19	3.22	0.48	N	E
135840	3.00	Great Dane	FN	Congenital	PM	C	6	YES	YES	NO	21.47	0.40	9.96	1.07	N	E
136586	5.50	Old English sheepdog	F	Gastrointestinal	Clin	C	1	NO	NO	NO	31.23	0.04	28.32	1.59	N	F
136521	12.25	Cocker spaniel	M	General Medicine	CP	C	5	NO	NO	NO	37.11	0.54	23.59	1.94	N	P

Appendix 36 (continued)

Case Number	Age (yrs)	Breed	Sex	Category	Method	Disease course	CDS	Suppressive therapy ?	Any drug therapy ?	Thyroid Therapy ?	total T_4	cTSH	free T_4	total T_3	TgAb	Outcome
136594	10.50	Retriever	F	Neurological	Clin	C	2	YES	YES	NO	5.99	0.08	12.72	1.26	N	P
133663	11.17	Crossbreed	M	Dermatological	CP	C	2	YES	NO	YES	72.72	0.01	62.38	1.95	N	P
132788	9.50	Bearded collie	F	General Medicine	Clin	C	4	YES	YES	NO	26.15	0.18	32.60	1.85	N	F
136593	0.83	Golden retriever	M	Orthopaedic	IMG	C	2	YES	YES	NO	4.70	0.01	6.20	0.73	N	P
136608	14.00	JRT	FN	Ophthalmological	HP	C	2	NO	NO	NO	8.93	0.30	9.20	1.64	N	F
136601	6.50	Bernese mountain dog	M	Neoplasia	HP	C	8	NO	YES	NO	31.85	0.41	40.45	2.12	N	P
136600	1.50	Golden retriever	M	Cardiovascular	IMG	C	1	NO	NO	NO	26.10	0.01	26.43	2.01	N	F
136624	10.00	German shepherd	FN	Neoplasia	IMG	A	10	NO	YES	NO	27.51		28.77	1.04	N	E
136714	4.50	Labrador	M	Neoplasia	HP	A	10	NO	NO	NO	7.62	0.10	17.23	0.24	N	E
134918	11.67	Border collie	FN	Neoplasia	HP	C	2	NO	NO	NO	21.16	0.34	14.79	1.62	N	P
121252	9.00	Rottweiler	F	Endocrine	PM	A	8	NO	YES	NO	9.51	0.11	12.24	0.67	N	E
136647	2.75	CKCS	F	Orthopaedic	Clin	C	3	NO	NO	NO	25.16	0.06	22.73	0.92	N	F
136105	6.17	Labrador	M	Infectious	CP	C	8	NO	YES	NO	14.14	0.12	17.15	1.18	N	P
136637	9.50	Terrier	M	Neoplasia	HP	C	3	YES	YES	NO	40.79	0.11	26.94	1.35	N	E
136636	3.00	GSD	F	Respiratory	PM	A	9	YES	YES	NO	4.70	0.09	3.22	0.29	N	E
136634	9.00	Old English sheepdag	M	Cardiovascular	IMG	C	5	NO	YES	NO	15.47	0.65	21.64	1.11	E	P
136650	8.00	GSD	FN	IM	Clin	C	1	NO	NO	NO	41.55	0.34	26.51	1.87	N	P
127335	8.67	CKCS	M	General Medicine	HP	C	5	NO	YES	NO	23.08	0.04	31.79	1.30	N	P
136660	7.50	Labrador	F	Neoplasia	CP	C	7	NO	YES	NO	4.70	3.65	8.05	0.40	N	E
136360	6.17	Samoyed	FN	Endocrine	CP	C	3	NO	YES	NO	25.32		33.42	1.29	N	P
999999	6.42	Cairn terrier	F	General Medicine	CP	A	2	NO	NO	NO	26.17		13.19	2.05	N	F
135513	1.42	Border collie	F	Orthopaedic	IMG	C	1	NO	NO	NO	40.26	0.04	17.97	2.17	N	F
136665	2.50	Trailhound	F	Respiratory	Clin	C	8	NO	YES	NO	9.50	0.04	22.53	1.37	N	P
136661	0.92	Golden retriever	F	Neoplasia	HP	C	1	NO	NO	NO	51.85	0.07	43.03	1.53	N	F
134241	5.00	Cocker spaniel	FN	Neurological	CP	C	1	NO	NO	NO	29.34	0.04	48.88	2.51	N	P
135906	5.83	Bullmastiff	FN	Neoplasia	HP	C	5	YES	YES	YES	5.23	0.37	46.41	0.67	N	P
136699	4.00	Beagle	FN	Orthopaedic	IMG	C	2	NO	NO	NO	32.02	0.05	29.63	1.82	N	F
113791	9.83	Labrador	M	Urogenital	IMG	C	2	NO	YES	NO	4.70	0.18	17.65	0.55	N	P
136705	6.83	Dalmation	M	Neurological	CP	C	1	YES	YES	NO	26.81	0.32	36.68	2.23	N	P
136715	9.00	Collie cross	FN	Unknown	HP	C	7	NO	YES	NO	7.26	0.14	14.97	0.83	N	P
136709	7.83	CKCS	M	Cardiovascular	IMG	C	5	NO	YES	NO	17.57	0.07	28.91	1.01	N	E

Appendix 36 (continued)

Case Number	Age (yrs)	Breed	Sex	Category	Method	Disease course	CDS	Suppressive therapy ?	Any drug therapy ?	Thyroid Therapy ?	total T_4	cTSH	free T_4	total T_3	TgAb	Outcome
136713	5.75	Irish water spaniel	M	Neoplasia	HP	A	6	NO	YES	NO	16.66	0.15	17.09	1.41	N	P
131178	8.42	Standard poodle	M	Orthopaedic	IMG	A	1	NO	NO	NO	14.99	0.27	18.62	1.35	N	F
136448	2.33	Boxer	FN	Orthopaedic	IMG	C	1	NO	NO	NO	30.88	0.28	31.37	1.30	N	F
136711	9.50	Crossbreed	FN	Neoplasia	HP	C	2	NO	YES	NO	21.60	0.08	23.88	1.21	N	P
136707	11.00	American cocker spaniel	FN	Ophthalmological	Clin	A	2	YES	YES	YES	11.85	0.02	12.93	1.35	N	P
135374	1.75	Border collie	F	General Medicine	CP	C	5	NO	YES	NO	13.69		23.54	1.50	N	P
136708	9.00	Cocker spaniel	F	Neoplasia	HP	C	3	NO	YES	NO	30.18	0.22	24.30	1.95	N	P
136704	11.00	Border collie	FN	Neoplasia	IMG	C	2	NO	YES	NO	16.41		22.68	0.24	N	P
136769	10.00	Pekinese	M	Trauma	IMG	A	3	NO	NO	NO	20.65	0.03	16.97	0.81	N	F
133934	7.08	Cairn terrier	M	Urogenital	CP	C	3	NO	NO	NO	15.50	0.31	20.38	1.65	N	P
136763	10.50	Retriever	M	Neoplasia	IMG	C	8	YES	YES	NO	4.70	0.04	12.84	0.22	N	E
131345	2.50	Crossbreed	F	Endocrine	CP	C	1	YES	YES	NO	49.82		33.25	3.28	N	P
136759	4.00	Akita	MN	Gastrointestinal	IMG	C	3	YES	YES	NO	9.72		14.98	0.85	N	P
136733	0.83	Standard poodle	M	Trauma	IMG	A	7	NO	NO	NO	22.74	0.05	47.11	0.74	N	F
136748	2.00	Bullmastiff	F	Neoplasia	HP	A	7	NO	NO	NO	24.00	0.15	35.82	1.09	N	E
136742	7.00	GSP	F	Orthopaedic	IMG	C	2	YES	YES	NO	13.28	0.18	20.49	1.08	N	P
136754	5.00	Great Dane	M	Neurological	CP	C	1	NO	YES	NO	19.36			1.64	E	P
134463	9.92	Pyranean mountain dog	M	Endocrine	CP	C	4	NO	NO	NO	12.29		25.54	1.62	N	P
136756	4.00	English springer spaniel	M	Urogenital	IMG	C	4	YES	YES	NO	11.27	0.07	24.34	0.74	E	F
136736	11.00	Crossbreed	M	Gastrointestinal	Clin	C	5	NO	NO	NO	13.40	0.21	18.91	1.77	N	F
135515	1.33	Retriever	M	Orthopaedic	IMG	C	2	NO	NO	NO	28.25	0.13	26.79	1.43	N	F
136407	11.17	Retriever	M	Neoplasia	HP	C	4	YES	YES	YES	10.07	0.05	12.45	0.99	N	F
136771	1.50	GSD	F	Orthopaedic	IMG	C	2	NO	NO	NO	18.96	0.08	29.98	1.08	N	F
136770	2.50	Labrador retriever	F	Neurological	CP	A	3	NO	NO	NO	22.94	0.09	28.97	1.23	N	P
136523	13.08	Collie cross	M	Dermatological	HP	C	2	NO	NO	NO	37.90	0.37	36.21	0.83	N	P
123670	15.00	Crossbreed	MN	Gastrointestinal	CP	A	7	YES	YES	NO	18.88	0.18	32.46	1.03	N	F
136851	9.83	Rough collie	FN	Neoplasia	PM	A	9	NO	YES	NO	19.48	0.07	27.86	0.88	E	E
136850	8.00	Cocker spaniel	MN	IM	HP	A	7	YES	YES	NO	4.70	0.09	18.17	0.14	N	P
136841	9.83	Lurcher cross	M	Gastrointestinal	Clin	C	3	NO	YES	NO	38.17	0.20	35.74	1.96	N	F
136842	9.00	Terrier	M	Gastrointestinal	CP	C	7	NO	YES	NO	4.70	0.15	7.21	1.87	N	P

Appendix 36 (continued)

Case Number	Age (yrs)	Breed	Sex	Category	Method	Disease course	CDS	Suppressive therapy ?	Any drug therapy ?	Thyroid Therapy ?	total T_4	cTSH	free T_4	total T_3	TgAb	Outcome
136163	0.92	Rottweiler	M	Endocrine	CP	C	5	YES	YES	NO	9.63		60.73	1.33	N	F
136873	9.00	Weimeranner	FN	Orthopaedic	IMG	C	1	YES	YES	NO	35.65	0.58	27.52	2.48	N	F
122811	16.50	Yorkshire terrier	M	Gastrointestinal	Clin	C	2	NO	NO	NO	42.29		54.66	2.72	N	P
136803	8.00	Crossbreed	MN	Neoplasia	IMG	C	2	NO	YES	NO	11.99	0.22	13.27	1.44	N	E
131513	8.17	Rottweiler	F	Neurological	CP	C	2	YES	YES	NO	4.70	0.16	3.22	1.12	E	P
136400	6.17	German shepherd	M	IM	Clin	C	1	NO	YES	NO	7.37	0.07	30.23	0.30	N	F
136822	6.00	Labrador retriever	M	Orthopaedic	IMG	C	2	NO	NO	NO	22.63	0.08	20.36	1.28	N	P
136811	0.50	Doberman	F	Orthopaedic	IMG	A	1	YES	YES	NO	14.78	0.02	20.98	1.40	N	F
132058	8.00	Rottweiler	MN	Orthopaedic	Clin	C	5	YES	YES	NO	16.42	0.10	17.40	1.42	E	F
136882	1.67	Retriever	M	Congenital	Clin	C	1	NO	YES	NO	19.70	0.08	26.43	2.18	N	F
136883	10.00	GSP	M	Ophthalmological	Clin	C	2	NO	YES	NO	6.82	0.23	10.86	1.06	N	F
136884	7.00	Boxer	FN	Neurological	IMG	C	5	NO	NO	NO	22.11	1.01	19.61	0.82	N	E
133058	8.50	German shepherd	M	Gastrointestinal	Clin	C	2	YES	YES	NO	7.94	0.41	26.77	2.11	N	P
136308	4.67	WHWT	M	Neoplasia	HP	C	4	NO	YES	NO	41.40	0.05	40.68	1.36	N	P
134354	9.42	Sheltie	FN	Gastrointestinal	IMG	C	3	NO	NO	NO	7.20	0.40	25.59	1.37	N	P
134489	10.83	Labrador	M	Neoplasia	HP	C	5	YES	YES	NO	27.03	0.24	19.16	1.61	N	P
137838	9.00	German shepherd	MN	IM	Clin	C	1	NO	NO	NO	21.91	0.43	29.44	2.05	E	F
136120	7.17	Crossbreed	MN	Neoplasia	HP	C	8	NO	YES	NO	42.47	0.03	27.76	1.34	N	E
137956	6.00	Newfoundland	FN	Urogenital	CP	C	3	NO	YES	NO	22.82	0.26	20.52	1.68	N	F
137114	4.25	Rhodesian ridgeback	M	Orthopaedic	IMG	C	1	NO	NO	NO	19.61	0.11	22.66	1.72	N	F
137928	0.33	German shepherd	M	Gastrointestinal	IMG	A	8	YES	YES	NO	38.15	0.08	37.48	1.11	N	E
137944	5.00	Boxer	F	Neurological	Clin	C	2	YES	YES	NO	32.29	0.35	30.98	2.34	N	P
137947	8.00	Labrador	MN	Neurological	Clin	A	4	YES	YES	NO	13.84	0.08	14.77	1.09	N	P
137961	9.00	Lhasa Apso	F	Ophthalmological	Clin	A	2	NO	YES	NO	41.92	0.06		3.44	N	F
137910	4.00	Labrador	F	Ophthalmological	CP	C	1	NO	NO	NO	33.41	0.25	24.41	1.40	N	P
137909	12.42	Miniature Poodle	MN	Unknown	Clin	C	3	YES	YES	NO	4.70	0.03	5.73	1.00	E	F
137932	2.00	Newfoundland	FN	Infectious	CP	A	2	NO	YES	NO	21.39	0.03	17.20	1.54	N	F
137935	13.00	WHWT	M	Neoplasia	IMG	C	2	NO	YES	NO	11.19	0.09	14.33	1.02	N	E
137945	5.00	German shepherd	M	IM	Clin	C	1	NO	NO	NO	36.14	0.10	41.39	1.39	E	F
137995	7.00	St Bernard	M	Cardiovascular	IMG	C	4	NO	NO	NO	9.81	0.25	14.81	0.76	N	P
137953	6.42	German shepherd	M	IM	Clin	C	1	NO	YES	NO	36.45	0.33	37.65	1.46	N	F
137158	13.33	Collie cross	FN	Neoplasia	HP	A	7	YES	YES	NO	13.94	0.25	19.52	1.38	N	F

Appendix 36 (continued)

Case Number	Age (yrs)	Breed	Sex	Category	Method	Disease course	CDS	Suppressive therapy ?	Any drug therapy ?	Thyroid Therapy ?	total T_4	cTSH	free T_4	total T_3	TgAb	Outcome
137934	12.00	Labrador	MN	Respiratory	Clin	C	9	NO	NO	NO	18.36	0.11	3.22	0.47	N	E
130613	12.33	Cairn terrier	FN	Ophthalmological	Clin	C	3	NO	YES	NO	12.97	0.46	18.42	0.98	E	F
137938	1.50	Labrador	M	Neurological	CP	A	6	YES	YES	NO	4.70	0.03	4.00	0.55	N	P
137937	6.92	Boxer	MN	Neoplasia	IMG	C	5	NO	YES	NO	18.68	0.14	22.94	1.53	N	P
137906	2.92	Cocker spaniel	FN	Dermatological	Clin	C	2	NO	NO	NO	23.72	0.11	29.27	1.06	N	P
138081	4.00	Collie cross	M	Trauma	IMG	A	6	YES	YES	NO	12.63	0.04	14.48		N	P
138015	5.00	Labrador	F	Neurological	CP	A	8	NO	NO	NO	7.35	0.05	20.24	0.62	N	E
137943	7.00	CKCS	F	Neurological	IMG	A	4	NO	YES	NO	30.07		26.42	1.78	N	P
138049	5.00	Retriever	F	Respiratory	IMG	C	3	YES	YES	NO	22.44	0.18	24.29	1.58	N	P
138082	11.00	WHWT	M	Neoplasia	HP	A	4	NO	NO	NO	37.05	0.32	25.97	2.16	N	F
138097	0.75	Boxer	MN	Neurological	CP	C	5	YES	YES	NO	18.18	0.04	30.46	1.48	N	F
138036	10.00	English setter	M	Soft Tissue	Clin	C	3	YES	YES	NO	17.44	0.18	19.60	1.30	E	F
138052	0.58	Labrador retriever	M	Neurological	CP	A	5	YES	YES	NO	20.46	0.05	35.99	1.37	N	F
135421	12.00	Border collie	M	Orthopaedic	IMG	C	2	NO	NO	NO	16.45	1.01	9.86	2.19	N	P
138029	7.00	Cocker spaniel	M	Gastrointestinal	IMG	C	2	NO	YES	NO	31.04	0.20	25.54	2.07	E	P
137137	10.25	JRT	M	Ophthalmological	Clin	C	1	NO	NO	NO	20.50	0.13	17.53	1.97	N	P
136953	15.00	Collie cross	FN	Orthopaedic	Clin	C	1	YES	YES	NO	30.31	0.39	20.98	2.15	N	F
137529	4.25	English springer	F	Ophthalmological	Clin	C	1	NO	NO	NO	22.61	0.35	20.05	1.87	N	F
138117	0.50	Bernese mountain dog	M	Ophthalmological	IMG	C	1	NO	YES	NO	31.69	0.16	28.45	2.13	N	P
138116	4.42	English springer	M	Cardiovascular	IMG	C	3	NO	YES	NO	31.55	0.04	37.31	2.76	N	P
138132	8.67	Crossbreed	M	Orthopaedic	IMG	C	3	YES	YES	NO	32.61	0.11	7.49	2.35	E	P
138144	13.00	Collie cross	FN	Respiratory	IMG	C	3	YES	YES	NO	24.26	0.10	26.23	1.51	N	P

SBT- Staffordshire bull terrier WHFT- wire haired fox terrier JRT- Jack russell terrier CKCS- Cavalier king Charles spaniel

Disease course A- acute; C- chronic CDS- Clinical disease score (see Chapter 8 for definition); IM- Immune mediated

TgAb results: N- negative; E-equivocal "Suppressive therapy" corresponds to therapies capable of suppressing thyroid function

Method (Diagnostic method) Clin- clinical opinion; CP- clinicopathology; HP- histopathology; IMG- imaging; PM- postmortem

Outcome D- died; E- euthanatised; P- partial recovery; F- full recovery (see Chapter 8 for definitions)

ABBREVIATIONS

%	Percent
±	Plus or minus
μ	Micro
μl	Microlitre
μU	Microunits
3,5-T_2	3,5-diiodothyronine
5-D	5-deiodinase
Ab	Antibody
ACTH	Adrenocorticotropin
AITD	Autoimmune thyroid disease
BSA	Bovine serum albumin
cpm	Counts per minute
c.v.	Coefficient of variation
cAMP	Cyclic adenosine monophosphate
CCH	Chromic chloride passive haemagglutination
CDS	Clinical disease score
cTSH	Canine thyroid stimulating hormone
DCM	Dilated cardiomyopathy
DIT	Diiodothyrosine
dl	Decilitre (100 mililitres)
DPR	Differential positive rate
DVCS	Department of Veterinary Clinical Studies
ECG	Electrocardiographic
EDTA	Tri-potassium ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
FSH	Follicle stimulating hormone
fT_4I	Free thyroxine index
g	Grams
GCH	Glutaraldehyde
GnRH	Gonadotropin releasing hormone
HDL	High density lipoproteins
HDL_2	High density lipoprotein-2
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
i.u.	International unit
IFCC	International Federation of Clinical Chemistry
IgG	Gammaglobulin
IRMA	Immunoradiometric assay
KBq	Kilo Becquerels
KC	The Kennel Club
kg	Kilogram
L	Litre
lbs	Pounds (weight)
LDL	Low density lipoproteins
LH	Leutenising hormone
LMN	Lower motor neurone
LoD	Limit of detection
mg	Milligrams

MHC	Major histocompatability complex
MIT	Monoiodotyrosine
ml	Millilitre
N	Normal (molar strength)
ng	Nanogrames
nmol	Nanomoles
NSAID	Non-steroidal anti inflammatory drugs
NSB	Non-specific binding
NTI	Non-thyroidal illness
o.d.	Optical density
o.r.	Odds ratio
°C	Degrees celcius
p	Statistical probability value
PBS	Phosphate buffered saline
PC	Personal computer
PEG	Polyethyleneglycol
PFMA	Pet Food Manufacturers Association
pmol	Picomoles
PTU	Propylthiouracil
QC	Quality control
RIA	Radioimmunoassay
ROC	Receiver operating characteristic
rT_3	3,3',5'-T_3 (reverse-T_3)
s.d.	Standard deviation
s.e.m.	Standard error of the mean
SIBO	Small Intestinal Bacterial Overgrowth
SIR	Semi-interquartile range
S.I.	Système International d'Unités
T_3	3,5,3'-Triiodothyronine
T_3Ab	T_3 autoantibodies
T_3-S	T_3 sulphates
T_4	3,5,3',5',tetraiodothyronine (thyroxine)
T_4Ab	T_4 autoantibodies
TBG	Thyroid hormone binding globulin
TBPA	Thyroid hormone binding prealbumin
TC	Total counts (of radioactivity)
TCH	Tanned cell haemagglutination
Tg	Thyroglobulin
TgAb	Thyroglobulin autoantibodies
THBI	Thyroid hormone protein binding inhibitors
THBR	Thyroid hormone binding ratio
THRT	Thyroid hormone replacement therapy
TMB	3,3',5,5'-tetramethylbenzidine
TPO	Thyroid peroxidase
TPOAb	Thyroid peroxidase autoantibodies
TRAb	Thyrotropin receptor antibodies
TRH	Thyrotropin releasing hormone
TSH	Thyroid stimulating hormone
UK	United Kingdom
USA	United States of America
VLDL	Very low density lipoproteins

W	Area under the ROC curve
5'-D	5'-deiodinase
3,3'-T_2	3,3'-diiodothyronine
^{125}I	^{125}radioiodine
^{131}I	^{131}radioiodine

Note that abbreviations for all clinicopathological parameters are detailed in Appendices 2 and 4.

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