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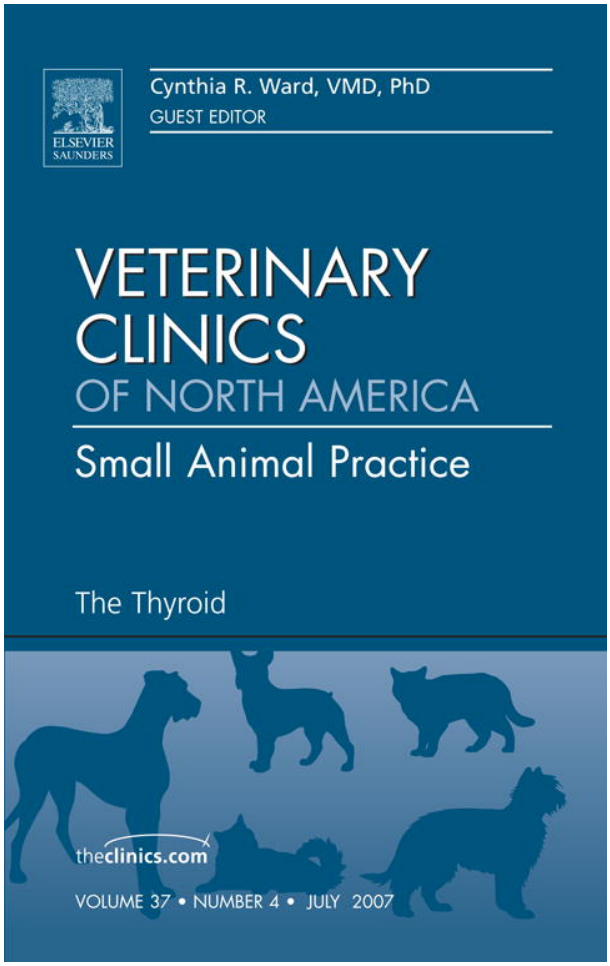

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Vet Clin Small Anim 37 (2007) 617–631
VETERINARY CLINICS
SMALL ANIMAL PRACTICE

Etiopathologic Findings of Canine Hypothyroidism

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It is apparent that the generation of thyroid hormones and the control mechanisms for their production and effects in target tissues are governed by many complicated processes. Failure of any one of the multiple required steps in thyroid hormone production, loss of hormonal trophic support from the pituitary, or destruction of the thyroid glands can result in hypothyroidism.

Although a range of possible causes of canine hypothyroidism exists, most cases arise from irreversible acquired thyroid gland disease. Only a small proportion of hypothyroidism cases result from nutritional, congenital, pituitary, hypothalamic, or reversible conditions. Hypothyroidism arising from failure of the thyroid glands is described as primary, that arising from pituitary failure as secondary, and that arising from the hypothalamus as tertiary.

ADULT-ONSET HYPOTHYROIDISM

Almost all the naturally occurring hypothyroidism in adult dogs is attributable to irreversible destruction of the thyroid glands. Histologically, primary hypothyroidism is divided into two main pathologic categories: lymphocytic thyroiditis or idiopathic thyroid degeneration (idiopathic follicular atrophy). Most estimates indicate an approximately 1:1 ratio of these two types of thyroid pathologic findings as the origin of clinical hypothyroidism in dogs.

Lymphocytic thyroiditis, also referred to as autoimmune thyroiditis, is characterized by lymphocytic infiltration of the thyroid glands with progressive destruction of thyroid follicles. The presence of this thyroid inflammation can be detected in serum by the measurement of antibodies to thyroid components (usually antithyroglobulin antibodies [TgAAs]). The progression of this disease process is slow, and extensive pathologic changes have occurred before the appearance of clinical signs of hypothyroidism. This condition is recognized

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0195-5616/07/$ – see front matter
doi:10.1016/j.cvsm.2007.05.002
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as a heritable trait [1–3]. Lymphocytic thyroiditis may sometimes present as a component of immune-mediated polyendocrinopathy [4,5].

Idiopathic thyroid degeneration is characterized by a loss of thyroid parenchyma, with replacement by adipose or fibrous tissue. The cause has not yet been defined, and it is likely that this category represents a collection of primary pathologic conditions, but there is evidence that at least a proportion of these cases represent an end-stage form of lymphocytic thyroiditis [5].

CONGENITAL HYPOTHYROIDISM

Congenital cases of hypothyroidism in the dog arise from defects in thyrotrophic support (absent or ineffective thyroid-stimulating hormone [TSH]), dyshormonogenesis of thyroid hormone, or thyroid gland development. In cases in which there is normal pituitary function, the failure of thyroid hormone production can be expected to result in goiter and histologic evidence of follicular hyperplasia. A nonsense mutation in the thyroperoxidase (TPO) gene causing hypothyroidism with goiter has been reported in Toy Fox Terries and related Rat Terriers [6,7]. Goiter is absent in most reports of congenital hypothyroidism. A lack of production of TSH is the suspected cause of juvenile hypothyroidism in Giant Schnauzer [8], Boxer [9], and Scottish Deerhound [10] dogs. Tertiary hypothyroidism has not been confirmed in dogs, although many reports of congenital hypothyroidism were published before the availability of the canine TSH assay, making the distinction between secondary and tertiary difficult to determine.

NATURAL HISTORY OF THYROID DISEASE

The pathway from completely healthy thyroid glands to glands that are sufficiently destroyed to result in such a degree of thyroid hormone deficiency that it becomes clinically apparent is probably not a short process in most circumstances of adult-onset hypothyroidism. The progression of lymphocytic thyroiditis from the earliest evidence of pathologic change to overt thyroid functional failure has been the subject of some study [5,11,12].

In the dog, the disease progresses through recognizable stages:

1. Subclinical (or silent) thyroiditis: the presence of focal and often peripheral lymphocytic infiltrates in the glands that have a normal histologic appearance otherwise; the only laboratory abnormality is TgAA in serum.
2. Antibody-positive subclinical hypothyroidism: if pathologic change encompasses more than 60% to 70% of the thyroid mass, we see a compensatory elevation of serum TSH concentration that stimulates the remaining portion of functional tissue to increase thyroid hormone production. Follicular epithelial cells demonstrate this stimulation histologically by a change from a cuboidal to columnar shape. Laboratory abnormalities in this stage include serum TgAA and increased TSH concentrations but normal concentrations of thyroxine (T_4) and triiodothyronine (T_3).

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3. Antibody-positive overt hypothyroidism: when nearly all functional thyroid tissue has been destroyed by inflammation, T_4 production cannot be maintained and the classic laboratory pattern of decreased total T_4, increased TSH, and positive antibody is found. It may be sometime thereafter before physical clinical signs are documented. In experimental settings in which functionally overt hypothyroidism has been induced by surgery or radiation, clinical signs took some time to develop and were not clearly apparent until more than a year later [13].
4. Noninflammatory atrophic hypothyroidism: there is some evidence to suggest that there is eventually replacement of thyroid tissue by fibrous and adipose tissue, with disappearance of inflammatory cells leading to a noninflammatory and atrophic histologic appearance. The absence of inflammation is likely to result in the disappearance of antibodies from the circulation over time. What contribution this end-stage of thyroiditis makes to the 50% of canine hypothyroidism that is antibody-negative (idiopathic) has yet to be defined.

The progression of idiopathic follicular atrophy attributable to causes other than end-stage thyroiditis has not yet been studied, because there is not yet a diagnostic test for the subclinical form. It has to be assumed that the disease progresses through similar functional stages, but the time scale is unknown.

The progression of thyroiditis to functional hypothyroidism is supported by long-term follow-up of affected dogs [11,12,14] and database studies of age distributions of the different functional and pathologic stages (Fig. 1). Not all cases progress to overt disease, however, and there is limited or slow progression in some.

ETIOPATHOLOGIC FINDINGS OF CANINE INFLAMMATORY THYROID DISEASE

Little is known about the initiators of canine thyroid disease, although recent work has laid the groundwork for further study and epidemiologic surveys hope to give some direction for suitable areas of investigation. Most of what we understand about the initiation of canine thyroid disease comes from studies in other species, including human beings, although the canine disease has recently been the subject of further investigation.

The pathologic findings of thyroiditis are predominantly lymphocytic and consist of B- and T-cell components. Two forms of chronic autoimmune thyroiditis are recognized in human medicine: goitrous autoimmune thyroiditis (Hashimoto's disease), which does not occur in dogs, and atrophic autoimmune thyroiditis, which is more similar to the chronic autoimmune thyroiditis of dogs. The pathologic findings in these conditions are similar, with the exception of goitrous enlargement in the former, and include focal or diffuse lymphoplasmacytic infiltration with macrophages. Lymphoid germinal centers are often seen in moderate and severe cases, as is basement membrane disruption, including ultrastructural abnormalities consistent with antibody or antigen complex deposits. Enlarged, metaplastic, oxyphilic follicular epithelial

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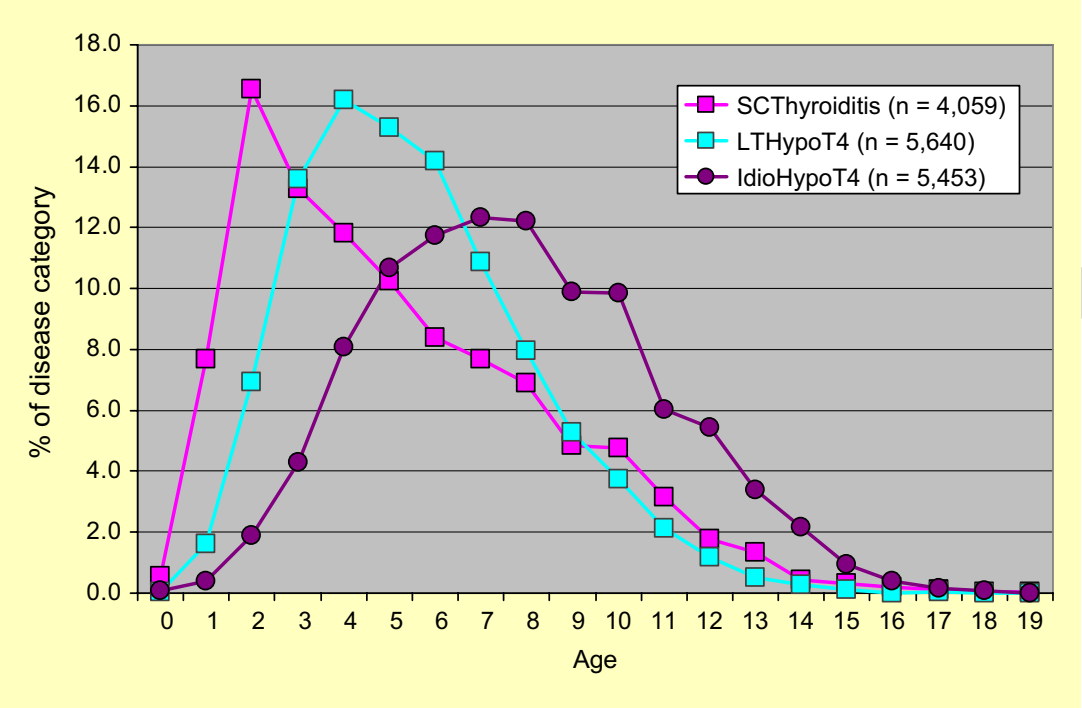

Fig. 1. Age distribution profiles for different categories of thyroid disease and dysfunction based on findings in 143,800 samples submitted for the investigation of thyroid disease in which an age was provided. IdioHypoT_4, TgAA-negative hypothyroidism; LTHypoT_4, TgAA-positive hypothyroidism; SC thyroiditis, subclinical TgAA-positive thyroiditis.

cells (Hürthle cells) with granular mitochondria-rich cytoplasm are also seen. In dogs, the pathologic findings of this condition have been described numerous times [11,15–23]. It is histologically identical to human chronic autoimmune thyroiditis, and histologic grading systems have been developed [15,23].

The immunologic and molecular pathogenesis of autoimmune thyroiditis in dogs has not yet been well characterized. Most of what is known about the condition has been learned from the induction of experimental disease and research of the human condition. Although thyroiditis has been studied in laboratory rodents and chickens and experimentally induced in dogs, it is not clear how well these models mimic naturally occurring disease. In human beings, the lymphocytic infiltrate contains B cells and mostly T cells. CD4 and cytotoxic CD8 T lymphocytes are present, and evidence exists for thyroid follicular cell destruction through antibody-dependent complement-mediated mechanisms and cytotoxic T cells [24]. Recent work has confirmed the proliferative responses of peripheral blood mononuclear cells to canine thyroglobulin in TgAA-positive hypothyroid dogs and suggests that a loss of self-tolerance in CD4+ cells is important in the pathologic findings of canine thyroiditis [25]. To what extent this immunologic phenomenon is an initiator rather than a consequence of the thyroid pathologic condition has yet to be understood.

Experimental thyroiditis and human autoimmune thyroiditis seem to be disorders of immunoregulation. Therefore, in the search for the underlying molecular abnormality in this condition, research effort has been focused on mechanisms of immunoregulation, particularly the contribution made by the human leukocyte antigen (HLA) complex. Some association has been documented between certain HLA subtypes and the presence of disease; however, to date, these associations have generally been weak. Work on the contributions of dog leukocyte antigen (DLA) subtypes to the disease in dogs is under investigation [26]. The genetics of the DLA have recently been investigated, and predisposing alleles have indeed been identified [27,28]. The DLA-DQA1*00101 allele seems to be particularly influential and is associated with an increased risk of hypothyroidism (overall odds ratio = 1.97; $P < .001$). Although especially prevalent in Doberman Pinschers, English Setters, and Rhodesian Ridgebacks (including unaffected individuals), this was not the case in other breeds, such as the Boxer. This is consistent with the predisposition associated with HLA subtypes.

In the investigation of potential nonimmunogenetic causes, such as mutations in the canine thyroglobulin gene or its promoter, no variations correlating with the presence of thyroiditis have been revealed [26], although canine thyroglobulin has now been cloned and sequenced [29], opening the possibility for further research in this area. The possibility that thyroiditis is induced in predisposed individuals by antigenic mimicry of thyroid antigens by viral or bacterial agents has been suggested. This possibility is supported by the protective effects of intestinal sterilization in experimental thyroiditis [30] and serologic evidence of recent infections in affected human patients [31,32]. *Yersinia enterocolitica* antibodies have been identified in human patients with Grave's disease [33] (a form of autoimmune thyroid disease in which anti-TSH receptor antibodies result in hyperthyroidism), and an increased frequency of antiretroviral antibodies has been found in human patients with autoimmune thyroiditis [34]. An alternative viral mechanism could be through the local induction of interferon-γ (IFNγ)–triggering H(D)LA expression by thyrocytes initiating an autoimmune process [34]. The contribution of immunoregulation in this disease is also inferred by the possible modulation of immunotolerance by oral feeding of thyroglobulin, after which some measures of thyroid autoimmunity can be ameliorated [35].

A protective effect of whole-body irradiation against familial lymphocytic thyroiditis in beagles, especially when administered at around 2 days of age, has been documented and was greatest in genetically predisposed dogs [1,2,36]. Whether this phenomenon is mediated through effects on the developing immune system or on thyroid gland structure or function requires further investigation.

The diversity of prevalence among breeds (Table 1) and several specific heritability studies [2,3,36,37] indicate the highly heritable nature of this condition, and further studies indicate that there is a breed influence on age and progression of the disease [5].

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Table 1
Twenty breeds with the highest and 20 breeds with the lowest prevalence of thyroglobulin antibody in 140,821 serum samples submitted for investigation of thyroid disease

Name	Total sera	TgAA-positive	Prevalence
English Setter	585	184	31%
Old English Sheepdog	368	86	23%
Boxer	2642	496	19%
Giant Schnauzer	263	49	19%
American Pit Bull Terrier	345	64	19%
Beagle	2452	449	18%
Dalmatian	1372	246	18%
German Wirehaired Pointer	112	20	18%
Maltese Dog	594	105	18%
Rhodesian Ridgeback	626	107	17%
Siberian Husky	1129	164	15%
American Staffordshire Terrier	151	24	16%
Cocker Spaniel	8576	1305	15%
Chesapeake Bay Retriever	509	74	15%
Tibetan Terrier	106	15	14%
Shetland Sheepdog	5765	813	14%
Golden Retriever	17782	2397	13%
Borzoi	266	35	13%
Brittany Spaniel	556	71	13%
Dachshund	3612	115	3%
Basset Hound	699	22	3%
Cairn Terrier	590	18	3%
Schnauzer (unspecified)	1257	38	3%
Wirehaired Fox Terrier	170	5	3%
Cavalier King Charles Spaniel	274	8	3%
Welsh Corgi (undetermined)	457	13	3%
Yorkshire Terrier	1178	33	3%
Norwegian Elkhound	263	7	3%
Belgian Tervuren	235	6	3%
Chihuahua	611	15	2%
Greyhound	1409	32	2%
Pekingese	407	9	2%
Boston Terrier	500	11	2%
Pomeranian	1301	26	2%
Irish Wolfhound	210	4	2%
Whippet	114	2	2%
Soft-Coated Wheaten Terrier	214	3	1%
Bichon Frise	657	8	1%
Miniature Schnauzer	828	10	1%

Overall TgAA prevalence in this study was 10%.

There has been controversy in recent years concerning the possible contribution that routine vaccination might make to the origin of thyroiditis in dogs. In one study, it seemed that there might be support for vaccination as an initiator of thyroid pathologic change. Scott-Moncrief and colleagues [38,39] reported an increase in circulating antibodies that reacted with thyroglobulin after repeated vaccination; however, further research by the same group failed to demonstrate an increased prevalence of thyroiditis in vaccinated beagles postmortem after a 5.5-year follow-up study [40].

The research experience in other species and in related immune-mediated disease has shown that the origins of thyroiditis in an individual animal are likely to be multifactorial. Using a large research database containing the results of 143,000 serum thyroid investigations and questionnaire studies, researchers at Michigan State University have explored how candidate predisposing factors, including breed, seasonality, and geography, contribute to the initiation of thyroid pathologic change.

In addition to identifying the prevalence of TgAA (as a marker for the prevalence of thyroiditis) across a range of breeds, these researchers have also noted a wide variation in the relative proportions of antibody-positive (thyroiditis) and antibody-negative (idiopathic atrophy) hypothyroidism across breeds. The widely reported overall average of 50:50 holds true; however, in some breeds, the contribution of thyroiditis is much greater or much less. In English Setters, for example, more than 80% of cases diagnosed with hypothyroidism were TgAA-positive, whereas less than 30% of hypothyroid Doberman Pinschers were antibody-positive (Table 2). These findings suggest a different rate or type of progression of thyroiditis or breed differences in predisposition to noninflammatory forms of thyroid disease.

Using age-distribution profiles similar to that in Fig. 1 on a breed-specific basis (Figs. 2 and 3), there is indeed some evidence to suggest that there may be different progression rates among breeds.

There may be a small contribution of season of the year to the occurrence of earliest evidence of thyroiditis. Of dogs with no laboratory evidence of thyroid dysfunction, the proportion with evidence of thyroiditis (positive TgAA) was highest in the summer (July, August, and September) and lowest in the fall (October, November, and December) (Table 3).

In a preliminary investigation of the influence of geography on the prevalence of thyroiditis in samples submitted to Michigan State University, some significant differences were observed. The prevalence of TgAA was significantly higher in samples submitted from North Dakota, Vermont, Wyoming, Minnesota, and Colorado compared with Michigan (range of odds ratios: 1.19–1.41; $P < .05$). The prevalence was significantly lower in samples from Massachusetts, Maryland, Virginia, North Carolina, Florida, South Carolina, Kentucky, Texas, West Virginia, Tennessee, and Alabama (range of odds ratios: 0.39–0.79; $P < .05$). There was no interaction with breed prevalence, but the underlying reasons (if any) for these observations have yet to be discovered.

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Table 2
Proportion of TgAA-positive results by breed in 11,606 serum samples from dogs with laboratory results consistent with hypothyroidism (restricted to breeds with >40 cases)

Breed	TgAA-negative hypothyroidism	TgAA-positive hypothyroidism	Total hypothyroidism	Proportion TgAA-positive
English Setter	12	61	73	84%
Chesapeake Bay Retriever	15	36	51	71%
Golden Retriever	475	1050	1525	69%
Rhodesian Ridgeback	15	27	42	64%
Boxer	93	166	259	64%
Siberian Husky	45	74	119	62%
Irish Setter	16	26	42	62%
Cocker Spaniel	451	683	1134	60%
Border Collie	31	44	75	59%
Dalmatian	110	152	262	58%
Maltese Dog	39	52	91	57%
American Pit Bull Terrier	19	25	44	57%
Shetland Sheepdog	303	395	698	57%
Beagle	240	276	516	53%
Australian Shepherd	31	32	63	51%
Mixed breed	1249	1286	2535	51%
Akita	22	22	44	50%
Great Dane	30	29	59	49%
Brittany Spaniel	36	33	69	48%
Scottish Terrier	31	26	57	46%
Malamute	36	30	66	45%
Samoyed	30	23	53	43%
Labrador Retriever	577	376	953	39%
Rottweiler	102	60	162	37%
Chow Chow	53	28	81	35%
Springer Spaniel	75	38	113	34%
German Shepherd Dog	101	50	151	33%
Shih Tzu	31	14	45	31%
Keeshond	34	15	49	31%
Doberman Pinscher	392	135	527	26%
Poodle	68	22	90	24%
Collie	95	26	121	21%
Pomeranian	33	9	42	21%
Dachshund	68	13	81	16%
Grand total	5680	5926	11606	51%

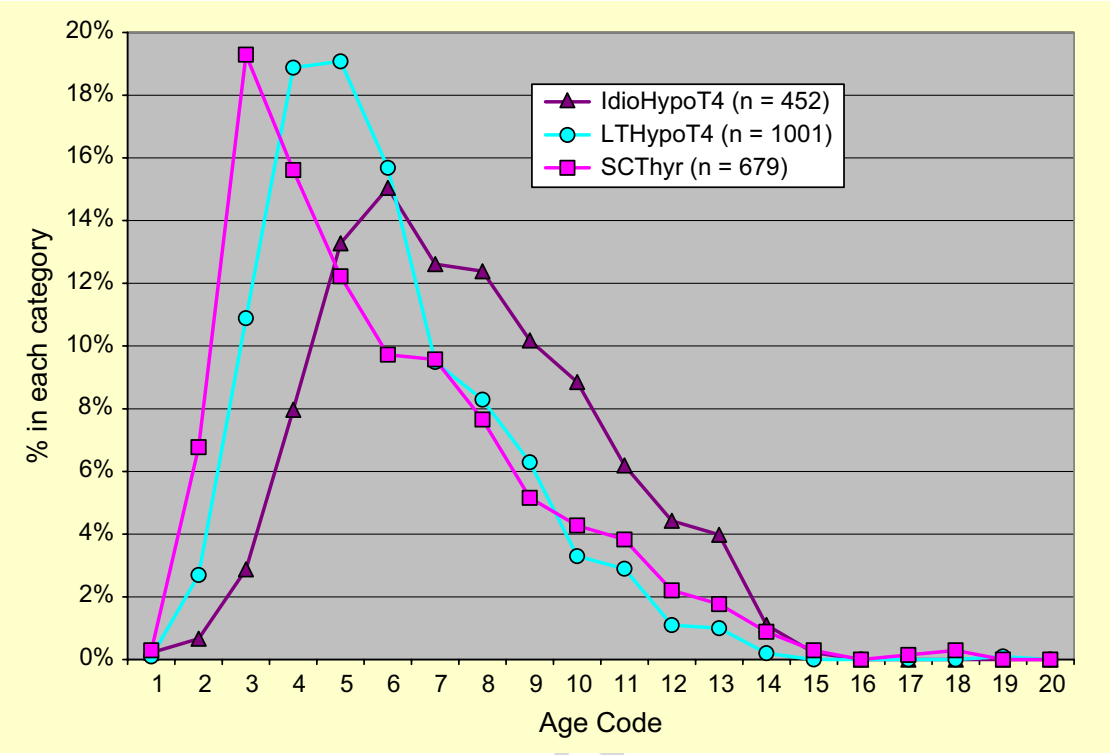

Fig. 2. Age distribution profiles for different categories of thyroid disease and dysfunction based on findings in 17,782 samples submitted from Golden Retrievers for the investigation of thyroid disease in which an age was provided. IdioHypoT_4, TgAA-negative hypothyroidism; LTHypoT_4, TgAA-positive hypothyroidism; SC thyroiditis, subclinical TgAA-positive thyroiditis.

LABORATORY DIAGNOSIS OF THYROIDITIS AND IMPLICATIONS FOR DIAGNOSIS OF HYPOTHYROIDISM

During the inflammatory process of lymphocytic thyroiditis, antibodies are released into the circulation. In the dog, these are predominantly reactive against thyroglobulin. In people, the most common antigen to which antibodies are detected in patients with thyroiditis is TPO. Studies of anti-TPO as part of the process of thyroiditis in dogs have yielded mixed results [14,41,42]. A recent report provides evidence that they may be part of the process [43], albeit that their presence is documented only in dogs that also have TgAA or thyroid hormone auto antibodies (THAAs). This study found that 17% of TgAA-positive serum samples also reacted with TPO.

The thyroglobulin molecule is large and complex and contains sites at which thyroid hormones are assembled, incorporated, and stored. The size and complexity of the thyroglobulin molecule is such that antibodies against it form a heterogenous group directed at several epitopic sites. Several different segments of the thyroglobulin molecule, including some hormonogenic sites, seem to have greater antigenicity than others [26,44], and a small number of tryptic peptides of canine thyroglobulin have been shown to react consistently with TgAA-positive serum samples from 10 hypothyroid dogs (43-, 32.5-, 31-, and possibly 25-kd fragments) [45], although other attempts have failed to find

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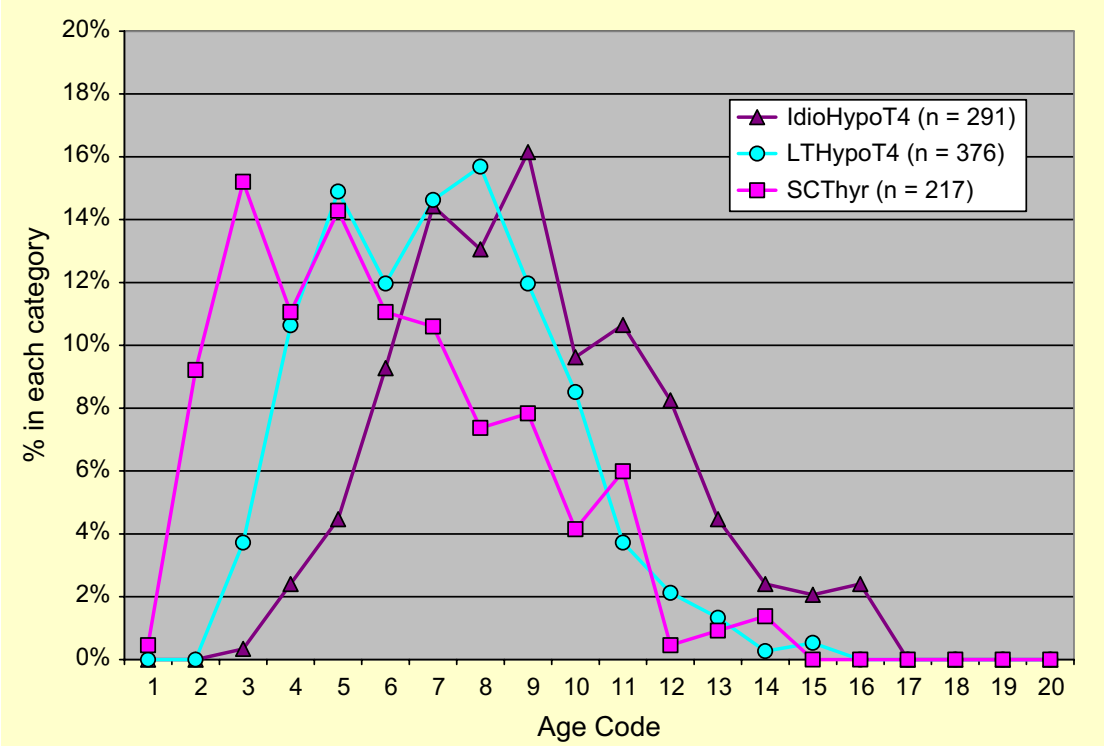

Fig. 3. Age distribution profiles for different categories of thyroid disease and dysfunction based on findings in 5765 samples submitted from Shetland Sheepdogs for the investigation of thyroid disease in which an age was provided. IdioHypoT_4, TgAA-negative hypothyroidism; LTHypoT_4, TgAA-positive hypothyroidism; SC thyroiditis, subclinical TgAA-positive thyroiditis. (*Data from* Michigan State University, East Lansing, Michigan, 2006.)

such fragment specificity in the dog [46]. Hormonogenic sites in canine thyroglobulin are conserved [29].

When an epitopic site includes a hormonogenic site, an antibody can be directed against a fragment that contains T_4 or T_3, creating an TgAA that cross-reacts with unbound T_3 or T_4. T_3 and T4 are not sufficiently large molecules to initiate an antibody themselves. The development of antibodies against epitopes that do not include hormonogenic areas results in TgAAs that do not

Table 3
Proportion of TgAA-positive results in 100,101 serum samples from euthyroid dogs by season

Season	Euthyroid TgAA-positive	Total euthyroid	Proportion TgAA-positive
January, February, March	850	19,345	4.39%
April, May, June	1084	23,722	4.57%
July, August, September	1465	29,885	4.90%
October, November, December	1072	27,149	3.95%
—	—	χ^2	31.19
—	—	*P*	.00000078

Table 4
Prevalence of thyroglobulin and thyroid hormone cross-reacting antibodies in different classes of serum from 143,800 samples submitted for investigation of thyroid disease

Category	Of 11,606 hypothyroid dogs	Of 5926 TgAA-positive hypothyroid dogs	Of 14,016 TgAA-positive dogs
TgAA	51%	100%	100%
Any THAA	30%	49%	39%
T_3AA	28%	46%	37%
T_4AA	8%	14%	11%
T_3AA and T_4AA	6%	11%	10%
TgAA but no THAA	26%	51%	61%
T_3AA but no T_4AA	21%	35%	27%
T_4AA but no T_3AA	2%	2%	2%
THAA but no TgAA	5%	—	—

cross-react with thyroid hormones. From Table 4, it can be seen that of dogs with circulating TgAAs, 37% have antibodies that cross-react with T_3 and 11% have antibodies that cross-react with T_4. Almost all dogs with anti-T_4 antibodies also have anti-T_3 antibodies, and approximately 50% of TgAA-positive serum samples do not react with thyroid hormones.

TgAAs that cross-react with free thyroid hormones (THAAs) are unlikely to have physiologic consequences in the circulation, given the tiny proportions of free (unbound) T_3 and free T_4. The presence of THAAs becomes important in the diagnostic laboratory when immunologic methods are used to measure serum concentrations of T_3 or T_4, however. Serum samples that contain TgAAs (THAAs) that cross-react with T_3 are described as T3 cross-reacting autoantibodies (T_3AA)-positive, and, similarly, those that cross-react with T_4 are T4 cross-reacting autoantibodies (T_4AA)-positive. Immunologic methods of thyroid hormone measurement depend on tightly controlled amounts of laboratory-derived antihormone antibody and labeled hormone. In the situation in which a patient sample brings it own antihormone antibodies to the reaction chamber, control of the reaction conditions is lost and false laboratory results are generated. In most assay systems, the effect of THAA is to cause a falsely higher measured concentration of the respective hormone. It is useful to note that this increase need not necessarily be greater than the laboratory reference range. In a few assay systems (eg, Michigan State University total T_3), a falsely lower value may be generated. The nature of the assay inaccuracy (falsely elevated versus falsely lowered) depends on the method used to separate radioligand bound to assay antibody from unbound radioligand, the so-called "separation step." If the dog's THAA becomes separated from the assay antibody, the calculated hormone concentration is falsely elevated. If the dog's

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THAA remains with the assay antibody, the calculated result is falsely lowered. Assays like as the free T_4 by equilibrium dialysis method, which removes the patient antibody (by dialysis) before the immunoassay step, are free from THAA interference.

The high proportion of T_3AA in hypothyroid dogs is the underlying reason why serum total T_3 measurement has not been found to be a useful test in the diagnosis of canine hypothyroidism. When T_3AA-positive animals are excluded, the diagnostic performance of T_3 is similar to the other measures of thyroid function (total thyroxine [TT_4], TSH, and free T4 by equilibrium dialysis [FT_4d]).

The diagnostic implication of the prevalence of T_4AA in hypothyroid dogs is that a normal or high TT_4 alone cannot be used conclusively to rule out a diagnosis of hypothyroidism. The addition of T_4AA (or TgAA) to a panel gives an indication of whether a normal serum TT_4 result can be believed.

The diagnostic implication of TgAA in the absence of evidence of thyroid dysfunction is that around 1 in 5 cases has progressive dysfunction within a year and 1 in 20 cases is hypothyroid [12].

Several TgAA assays have been described in the literature [47–50], but many recent reports, including the data presented in this article, have used a commercially available canine TgAA ELISA (Oxford Biomedical Research, Oxford,

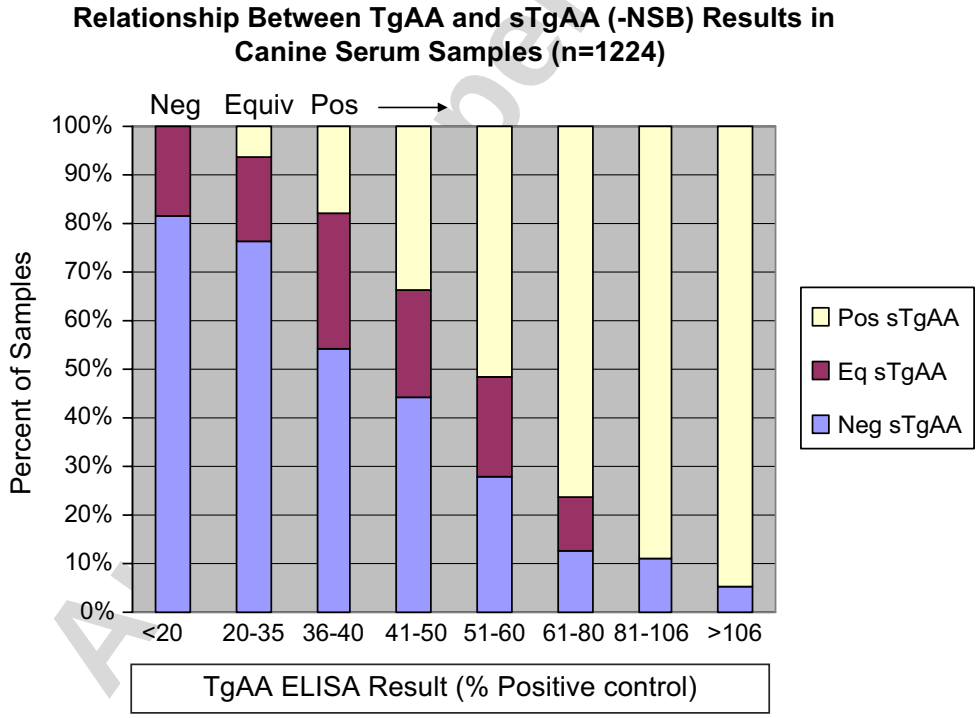

Fig. 4. Classification of direct thyroglobulin ELISA results with the outcome from adjustment for nonspecific binding in selected canine serum samples. Direct TgAA results are on the ordinate and expressed as the percentage of positive control (<20 is negative, 20–35 is equivocal, and >35 is positive). The vertical bars reflect the reclassification of the same samples after adjustment for nonspecific binding. (*Data from* Michigan State University, East Lansing, Michigan, 2006.)

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Michigan). This assay has undergone development during recent years to reduce the rate of false-positive and equivocal results. In the past, results from this assay were reported as patient optical density as a percentage of negative control optical density. Now, results can be expressed as percentage of a standardized positive control, and nonspecific binding ELISA plates (which lack thyroglobulin in plate wells) are also now provided to reduce the effect of IgG titers unrelated to TgAA. Some of the initial concern about TgAA becoming borderline positive in the months after vaccination [38,39] may have been attributable to increased nonspecific IgG binding, and the modifications to the assay should have now improved the confidence in positive TgAA results. In the evolution of setting cutoff values for negative, equivocal, or positive TgAA results, it seems that current guidelines maximize diagnostic sensitivity of detecting positive autoantibodies. When the direct TgAA ELISA and the nonspecific binding modification are run on the same sample, discordant results most often occur in slightly increased direct ELISA results that become equivocal or negative when adjusted for nonspecific binding (Fig. 4).

Acknowledgments

The authors gratefully acknowledge Mark Bowman and Ryan Lundquist for their assistance in manipulating and interrogating the large data sets reported in the article. They also acknowledge the clients of the Diagnostic Center for Population and Animal Health for the provision of clinical samples and demographic information with which epidemiologic studies are performed. Finally, they acknowledge the technical staff of the endocrine section for their expertise in performing the assays.

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