ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

DOTTORATO DI RICERCA IN

Scienze Veterinarie

Ciclo XXXVI

Settore Concorsuale: 07/H4

Settore Scientifico Disciplinare: VET/08

New insights on clinical features, clinicopathological abnormalities, diagnosis, treatment, and monitoring of canine Cushing's Syndrome

Presentata da: **Dott.ssa Stefania Golinelli_**

Coordinatore Dottorato

Prof.ssa Carolina Castagnetti

Supervisore

Prof. Federico Fracassi

Esame finale anno 2023

In this days when science is clearly in the saddle and
when our knowledge of disease
is advancing at a breathless pace,
we are apt to forget that not all can ride and
that he also serves who waits and who applies
what the horseman discovers (Harvey Cushing)

Contents

Abstract ***1***

Chapter 1 ..... ***1***

Aims and scope of the thesis 1

Chapter 2 ..... ***7***

General introduction 7

Chapter 3 ..... ***17***

Clinical features of muscle stiffness in 37 dogs with concurrent naturally occurring hypercortisolism 17

Chapter 4 ..... ***29***

Calcium and phosphate homeostasis in dogs with newly 29

diagnosed naturally occurring hypercortisolism 29

Chapter 5 ..... ***44***

Diagnosis of naturally-occurring hypercortisolism by primary care veterinarians: a Western European survey 44

Chapter 6 ..... ***56***

Comparison of methods to monitor dogs with hypercortisolism treated with trilostane 56

Chapter 7 ..... ***73***

Evaluation of clinical, ultrasonographic, and clinicopathological findings in dogs with pituitary-dependent hypercortisolism 73

and poor trilostane response 73

Chapter 8 ..... ***86***

Dopamine and somatostatin receptors and filamin A expression in normal pituitaries and corticotroph adenomas in dogs 86

Chapter 9 ..... ***89***

Addition of cabergoline to trilostane treatment for dogs with pituitary-dependent hypercortisolism 89

Chapter 10 ..... ***92***

Summarizing discussion and conclusions 92

Final report ..... *Errore. Il segnalibro non è definito.*

Abstract

The current thesis addresses different clinical, diagnostic, and therapeutic aspects of canine hypercortisolism (HC) or Cushing's syndrome, an endocrine disorder in dogs with an incidence of 1–2 cases per 1000 dogs per year. First, the thesis provides a comprehensive review of HC, covering clinical signs, clinicopathological abnormalities, diagnostic approaches, and treatment and monitoring options. Then, the present thesis focuses on muscle stiffness, a particular and poorly understood clinical sign associated with HC. Severe muscle stiffness (SMS) is characterized by persistent bilateral muscle contraction in various leg regions. The thesis also displays a thorough analysis of a larger cohort experiencing concurrent HC and SMS, providing insights into signalment, presentation, treatment strategies, and outcomes. The results of the analysis pointed out that muscle stiffness is a rare condition associated with HC. It seems to involve only PDH dogs and does not affect the life expectancy of these dogs.

Not only are specific and uncommon clinical manifestations linked to hypercortisolism (HC) in veterinary cases, but there are also clinicopathological abnormalities that remain inadequately comprehended. Specifically, the intricate relationship between cortisol and calcium homeostasis lacks clarity in the current understanding. Accordingly, the thesis delves into the impact of HC on calcium and phosphate metabolism in dogs. It evaluates circulating concentrations of whole parathyroid hormone (PTH), 25-hydroxyvitamin D (25-(OH)D), calcitriol, and fibroblast growth factor-23 in dogs with naturally occurring hypercortisolism and healthy dogs, examining their association with calcium and phosphate homeostasis.

The study results revealed that dogs with HC exhibit elevated serum phosphate concentrations, urinary fractional excretion of calcium (FECa), and PTH concentrations in comparison to the control group. These findings confirm the impact of cortisol on calcium balance.

The clinical signs and laboratory abnormalities point towards the suspicion of hypercortisolism, but specific hormonal tests are necessary for a conclusive diagnosis. However, diagnostic tests for HC currently have limitations, often yielding false-positive or false-negative results, and there is no established gold standard test. Consequently, diagnosing HC is a complex procedure that requires a meticulous interpretation of clinical signs, clinicopathological abnormalities, imaging findings, and results from endocrine tests. The careful selection of cases for specific endocrine tests is imperative to enhance diagnostic accuracy. A survey conducted during the Ph.D. period sheds light on the diagnostic methods used by Western European primary care veterinarians for HC showing testing protocols differ among WEPCVs. Nearly 60% of respondents may screen for HC in dogs even in the absence of consistent clinical signs, leading to concerns about potential overdiagnosis. Some WEPCVs do not attempt differentiation, potentially impacting management strategies and prognosis. The infrequent referral of cases to specialists indicates that HC is predominantly managed in first-opinion practices. These findings highlight the need for additional education among WEPCVs.

Once the diagnosis is established, it is important to choose the most suitable treatment for HC for each patient**.** Trilostane emerges as the medical treatment of choice for pituitary-dependent hypercortisolism (PDH). However, the most effective monitoring methods for trilostane treatment are still a subject of debate. The analysis aimed, therefore, to evaluate and compare 12 potential methods for monitoring trilostane treatment, with the goal of identifying clinical control in dogs categorized as well-controlled, undercontrolled, and unwell. The results emphasized that the clinical picture remains the gold standard for monitoring trilostane treatment, and none of the 12 analyzed methods can serve as an alternative but only as additional support.

While trilostane achieves various treatment goals, approximately 10-15% of dogs do not experience improvement and, currently, the reason for this is unknown. The study conducted provided a detailed comparison of clinical, ultrasonographic, and clinicopathological findings between dogs responding favorably and those with a poor response to trilostane treatment. Upon diagnosis, alopecia, alanine aminotransferase (ALT), creatinine, endogenous ACTH, different

cortisol concentrations, and ultrasound-detected adrenomegaly were found to be associated with a poor response to trilostane treatment.

The most effective strategy for managing PDH ideally focuses on directly addressing the pituitary tumor. Hypophysectomy is considered the gold standard for PDH treatment in humans and also in dogs. However, hypophysectomy is exclusively offered in large veterinary centers equipped with an established team of experienced surgeons, anesthetists, critical care specialists, and endocrinologists, with high initial costs. Consequently, the associated high initial costs and the limited availability of centers equipped to perform this procedure restrict owners' options for this treatment. Dopamine and somatostatin have emerged as crucial targets in the search for drugs that specifically target the pituitary and could replace hypophysectomy, considering their inhibitory functions within the gland. Another investigation analyzed the mRNA expression of various dopamine and somatostatin receptor subtypes and actin-binding protein filamin A in canine normal adenohypophysis (NAs) and canine adenomas (Cas). The mRNA of dopamine receptor D2 (DRD2) and somatostatine receptor 2 (SSTR2) was identified in the majority of CAs, albeit at lower levels compared to normal adenohypophysis NAs. The protein FLNA, crucial for the expression and signaling of DRD2 and SSTR2, was present in all CAs, indicating potential pathway activation. Cabergoline, a DRD2 agonist, has been employed in vivo in dogs with PDH, demonstrating an efficacy of 43% in reducing clinical signs, urine cortisol:creatinine ratio, endogenous ACTH, and even pituitary tumor size. Despite its reported efficacy, cabergoline has not been used in conjunction with trilostane. The last study assessesed, therefore, the impact of adding cabergoline to trilostane in controlling the clinical signs of PDH and potentially inhibiting or reducing the growth of pituitary tumors. Results of the study show an influence of cabergoline on pituitary tumor growth but not on clinical signs’ improvement.

Chapter 1

Aims and scope of the thesis

Hypercortisolism or Cushing's syndrome, was initially described by the neurosurgeon Harvey Cushing in 1932 in humans.[1] According to the European Society of Veterinary Endocrinology (ESVE) ALIVE project the definition of Cushing's syndrome is: "the umbrella term for a range of clinical syndromes that is caused by a chronic excess of glucocorticoid activity, which can be due to a range of endogenous or exogenous steroid hormones".[2]
Naturally occurring hypercortisolism (HC) is a prevalent endocrine disorder in dogs, with an incidence of 1–2 cases per 1000 dogs per year.[3-4] In 80–85% of cases, the etiology is attributed to an adrenocorticotrophic hormone (ACTH)-secreting pituitary adenoma, termed pituitary-dependent hypercortisolism (PDH). The remaining 15–20% typically stems from a cortisol-secreting adrenocortical tumor, often classified as an adrenocortical carcinoma. Uncommon causes of HC in dogs include ectopic ACTH syndrome and food-dependent hypercortisolism.[5]
In the introduction section of this thesis (**Chapter 2**), a comprehensive review is presented, covering clinical signs with a specific emphasis on rare manifestations, clinicopathological abnormalities with a particular focus on adrenal secondary hyperparathyroidism, diagnostic approaches, and treatment and monitoring options. Special attention is given to trilostane and alternative pituitary-targeting drugs such as cabergoline.

The predominant clinical manifestations of HC include polyuria and polydipsia (PU/PD), polyphagia, thin skin, excessive panting, bilaterally symmetrical truncal hair loss, and muscle weakness. The muscle weakness, resulting from chronic glucocorticoid excess, likely contributes to the observed "pot belly" appearance and exercise intolerance frequently observed in dogs with HC.[5] Severe muscle stiffness (SMS) is an uncommon occurrence in dogs with HC; when present, it is characterized by persistent bilateral muscle contraction in the thoracic legs, pelvic legs, or all four legs.
This combination of SMS in dogs with HC has been clinically termed "Cushing's myotonia," and fewer than 20 cases of dogs with both HC and SMS have been documented.[6-11] The pathogenesis, treatment options, and short- and long-term prognosis for dogs with HC and SMS remain unclear. A comprehensive analysis of a larger cohort of dogs experiencing concurrent HC and SMS is presented in **Chapter 3,** providing insights into signalment, presentation, treatment strategies, and outcomes.

Calcium-containing urolithiasis is a potential but infrequent finding of HC, indicating a potential influence of this condition on calcium balance.[12] Previous studies have not identified significant differences in total and ionized calcium (iCa) concentrations between dogs with HC and their healthy counterparts.[13-16] Hyperphosphatemia is a common occurrence in dogs with PDH, often accompanied by elevated serum parathyroid hormone (PTH) concentrations—a condition previously termed adrenal secondary hyperparathyroidism.[13-16] Moreover, in dogs newly diagnosed with PDH, hyperphosphatemia serves as an independent negative prognostic factor for survival, though the underlying cause of this association remains unclear.[17] Dogs with PDH exhibit lower urinary phosphate excretion and higher urinary calcium excretion compared to those without hypercortisolism.[14] The mechanism underlying these alterations has not yet been elucidated, for this reason, and to explore the potentially involved mechanism, **Chapter 4** delves into the exploration and evaluation of factors regulating calcium and phosphate homeostasis in dogs affected by hypercortisolism.

The existing diagnostic tests for HC have notable limitations, often yielding false-positive or false-negative results, and there is no universally accepted gold standard test. Consequently, diagnosing HC is a multifaceted process that requires careful consideration of clinical signs, clinicopathological abnormalities, imaging findings, and results from endocrine tests. Strategic selection of appropriate cases for specific endocrine tests is paramount to enhance diagnostic accuracy. Common diagnostic tests for investigating HC encompass the low-dose dexamethasone suppression test (LDDST), the

adrenocorticotropic hormone stimulation test (ACTHst), and the urine corticoid-to-creatinine ratio (UCCR).[5] The choice of the diagnostic approach for dogs suspected of HC is likely influenced by individual factors, including clinical expertise, experience, and personal preferences. A study examining diagnostic protocols employed by primary care veterinarians in the United Kingdom found that among 191 dogs diagnosed with HC, 95.3% underwent ACTHst, 33% underwent LDDST, and 27.8% underwent UCCR.[4] Differentiation of the origin of HC was infrequently conducted. The testing and differentiation protocols used by Western European primary care veterinarians (WEPCVs) are largely unknown. To shed light on the current diagnostic methods utilized by WEPCVs for HC, a survey was conducted (**Chapter 5**).

Trilostane is the medical treatment of choice for treating PDH. The drug exerts its effects by competitively inhibiting the steroidogenic enzyme 3β-hydroxysteroid dehydrogenase (3βHSD), a crucial player in the biosynthesis of all adrenocortical hormones. Through this action, trilostane disrupts the production of cortisol and aldosterone, triggering a cascade of hormonal responses.[5]

For the effective management of HC with trilostane, regular and frequent monitoring is essential. Over the past decade, considerable efforts have been dedicated to identifying the optimal approach for monitoring trilostane therapy, however, none of the methods investigated were reliable .[18-24] Regardless of the chosen method, the evaluation of clinical signs serves as the initial step.

Among the various monitoring methods, the commonly employed approach involves the use of the ACTH-stimulation test. This test assesses the adrenal glands' ability to secrete cortisol in response to stimulation, serving as an indicator of cortisol reserve. Despite its widespread use, the ACTHst has not been validated as a definitive monitoring tool for trilostane therapy. Over the years, several other potential monitoring methods have been proposed, but a comprehensive comparison among them is lacking.[18-24] Therefore, the study presented in **Chapter 6** aims to evaluate and compare the effectiveness of 12 potential methods for monitoring trilostane treatment, with the goal of accurately and objectively identifying clinical control in dogs categorized as well-controlled, undercontrolled, and unwell.

Trilostane effectively achieves various treatment goals, excluding the complete elimination of the underlying source. Studies have reported a gradual and variable control of clinical signs, with successful control observed in 50% to 100% of treated dogs within a few weeks. After several months of treatment, over 75% of cases in published studies showed partial to complete control of clinical signs.[25-33]

Nevertheless, approximately 10-15% of dogs treated with trilostane do not experience improvement in the clinical picture and clinicopathological variables.[5] Factors predicting trilostane efficacy have not been assessed to date. Evaluating the likelihood of a dog responding to the drug's action can be valuable for veterinarians and owners, especially considering the potential expense and rigorous monitoring requirements during the initial treatment months. **Chapter 7** provides a detailed comparison of various clinical, ultrasonographic, and clinicopathological findings between dogs exhibiting a favorable clinical response and those demonstrating a poor response to trilostane treatment.

The optimal approach to managing PDH ideally involves addressing the pituitary tumor directly. Dopamine and somatostatin have emerged as significant targets in the quest for drugs that specifically target the pituitary, given their inhibitory functions within the gland.

This emphasis on pituitary-directed drugs centers around three primary receptor subtypes: dopamine receptor subtype 2 (DRD2), somatostatin receptor subtype 2 (SSTR2), and somatostatin receptor subtype 5 (SSTR5). In the context of canine corticotroph adenomas, the predominant receptor subtype expressed is SSTR2, while DRD2 and, notably, SSTR5 are expressed at considerably lower levels.[34-35] Additionally, in humans, the actin-binding protein filamin A (FLNA) is

essential for the expression and signaling of SSTR2, SSTR5, and dopamine receptor 2.[36-37] **Chapter 8** aims to evaluate the mRNA expression of various dopamine and somatostatin receptor subtypes and FLNA in canine normal adenohypophysis (NAs) and canine adenomas (CAs).

Cabergoline, a DRD2 agonist, has been employed in dogs with PDH. By blocking DRD2, cabergoline has demonstrated its ability to decrease ACTH concentration and reduce tumor size in humans. Notably, in vivo experiments have yielded positive outcomes in dogs as well, with 43% of PDH-afflicted dogs exhibiting favorable responses to cabergoline treatment. These responses include the resolution of clinical signs, a decrease in ACTH concentration, and a reduction in pituitary tumor size.[38]

Despite its efficacy, cabergoline has not been used in conjunction with trilostane. It could be beneficial to combine a highly effective drug, such as trilostane, for controlling clinical signs with a medication capable of inhibiting pituitary tumor growth. For this reason, **Chapter 9** assesses the impact of adding cabergoline to trilostane in controlling the clinical signs of PDH and potentially inhibiting or reducing the growth of pituitary tumors.

The results, implications, limitations, and future perspectives of the studies included in this thesis are discussed in **Chapter 10**.

References

1. Cushing H. The basophil adenomas of the pituitary body and their clinical manifestations (pituitary basophilism). *Ann R Coll Surg Engl.* 1969;44:180-181.

2. ALIVE goals of treatment of naturally-occurring Cushing's syndrome. 2021 https://www.esve.org/alive/search.aspx.

3. Willeberg P, Priester W. Epidemiological aspects of clinical hyperadrenocorticism in dogs (canine Cushing's syndrome). *J Am Anim Hosp Assoc.* 1982;18:717–723.

4. O'Neill DG, Scudder C, Faire JM, et al. Epidemiology of hyperadrenocorticism among 210,824 dogs attending primary-care veterinary practices in the UK from 2009 to 2014. *J Small Anim Pract.* 2016;57(7):365–73.

5. Behrend EN. Canine hyperadrenocorticism. In: Feldman EC, Nelson RW, Reusch CE, et al., eds. Canine and Feline Endocrinology. 4th ed. St. Louis, MO: Elsevier Saunders; 2015:377-451.

6. Griffiths IR, Duncan ID. Myotonia in the dog: a report of four cases. *Vet Rec.* 1973;93:184-188.

7. Duncan ID, Griffiths IR, Nash AS. Myotonia in canine Cushing's disease. *Vet Rec.* 1977;100:30-31.

8. Greene CE, Lorenz MD, Munnell JF, et al. Myopathy associated with hyperadrenocorticism in the dog. *J Am Vet Med Assoc.* 1979;174:1310-1315.

9. Swinney GR, Foster SF, Church DB, et al. Myotonia associated with hyperadrenocorticism in two dogs. *Aust Vet J.* 1998;76:722-724.

10. Nagata N, Yuki M. Long-term outcome of myotonia associated with hyperadrenocorticism in 2 dogs. *Can Vet J*. 2015;56:931-933.

11. Nam S, Kang BT, Song KH, Seo KW. Long-term follow up of refractory myotonia associated with hyperadrenocorticism in a Maltese dog. *J Vet Clin.* 2020;37:273-277.

12. Hess RS, Kass PH, Ward CR. Association between hyperadrenocorticism and development of calcium-containing uroliths in dogs with urolithiasis. *J Am Vet Med Assoc.* 1998;212:1889-1891.

13. Ramsey IK, Tebb A, Harris E, Evans H, Herrtage ME. Hyperparathyroidism in dogs with hyperadrenocorticism. *J Small Anim Pract.* 2005; 46:531-536.

14. Tebb AJ, Arteaga A, Evans H, Ramsey IK. Canine hyperadrenocorticism: effects of trilostane on parathyroid hormone, calcium and phosphate concentrations. *J Small Anim Pract.* 2005;46:537-542.

15. Fracassi F, Malerba E, Furlanello T, Caldin M. Urinary excretion of calcium and phosphate in dogs with pituitary-dependent hypercortisolism: case control study in 499 dogs. *Vet Rec.* 2015;117:625.

16. Mooney CT, Shiel RE, Sekiya M, et al. A preliminary study of the effect of hyperadrenocorticism on calcium and phosphate concentrations, parathyroid hormone and markers of bone turnover in dogs. *Front Vet Sci.* 2020;7:1-9.

17. Fracassi F, Corradini S, Floriano D, et al. Paper: prognostic factors for survival in dogs with pituitary-dependent hypercortisolism treated with trilostane. *Vet Rec.* 2015;176:49.

18. Macfarlane L, Parkin T, Ramsey I. Pre-trilostane and three-hour post trilostane cortisol to monitor trilostane therapy in dogs. *Vet Rec.* 2016;179:597-601.

19. Arenas Bermejo C, Pérez Alenza D, García San José P, et al. Laboratory assessment of trilostane treatment in dogs with pituitary dependent hyperadrenocorticism. *J Vet Intern Med*. 2020;34:1413-1422.

20. McGrotty YL, Arteaga A, Knottenbelt CM, et al. Haptoglobin concentrations in dogs undergoing trilostane treatment for hyperadrenocorticism. *Vet Clin Pathol.* 2005;34:255-258.

21. Galac S, Buijtels JJ, Kooistra HS. Urinary corticoid:creatinine ratios in dogs with pituitary-dependent hyperadrenocorticism during trilostane treatment. *J Vet Intern Med.* 2009;23:1214-1219.

22. Arteaga A, Dhand NK, McCann T, et al. Monitoring the response of canine hyperadrenocorticism to trilostane treatment by assessment of acute phase protein concentrations. *J Small Anim Pract.* 2010;51:204-207.

23. Cook AK, Bond KG. Evaluation of the use of baseline cortisol concentration as a monitoring tool for dogs receiving trilostane as a treatment for hyperadrenocorticism. *J Am Vet Med Assoc*. 2010;237: 801-805.

24. Burkhardt WA, Boretti FS, Reusch CE, Sieber-Ruckstuhl NS. Evaluation of baseline cortisol, endogenous ACTH, and cortisol/ACTH ratio to monitor trilostane treatment in dogs with pituitary-dependent hyperadrenocorticism. *J Vet Intern Med.* 2013;27:919-923.

25. Ruckstuhl NS, Nett CS, Reusch CE. Results of clinical examinations, laboratory tests, and ultrasonography in dogs with pituitary-dependent hyperadrenocorticism treated with trilostane. *Am J Vet Res* 2002;63:506–512.

26. Galac S, Buijtels JJCWM, Mol JA, Kooistra HA. Effects of trilostane treatment on the pituitary-adrenocortical and renin-aldosterone axis in dogs with pituitary-dependent hypercortisolism. *Vet J*. 2008, doi:10.1016/j.tvjl.208.10.007

27. Braddock JA, Church DB, Robertson ID, Watson AD. Trilostane treatment in dogs with pituitary-dependent hyperadrenocorticism. *Aust Vet J.* 2003;81:600–607.

28. Augusto M, Burden A, Neiger R, Ramsey I. A comparison of once and twice daily administration of trilostane to dogs with hyperadrenocorticism. *Tierarztl Prax K H.* 2012;40:415–424.

29. Arenas C, Melian C, Perez-Alenza MD. Evaluation of 2 trilostane protocols for the treatment of canine pituitary-dependent hyperadrenocorticism: Twice daily versus once daily. *J Vet Intern Med.* 2013;27:1478-1485.

30. Cho KD, Kang JH, Chang D, Na KJ, Yang MP. Efficacy of low-and high-dose trilostane treatment in dogs (< 5 kg) with pituitary-dependent hyperadrenocorticism. *J Vet Intern Med.* 2013;27:91–98.

31. Alenza DP, Arenas C, Lopez ML, Melian C. Long-term efficacy of trilostane administered twice daily in dogs with pituitary-dependent hyperadrenocorticism. *J Am Anim Hosp Assoc.* 2006;42:269–276.

32. Vaughan MA, Feldman EC, Hoar BR, Nelson RW. Evaluation of twice-daily, low-dose trilostane treatment administered orally in dogs with naturally occurring hyperadrenocorticism. *J Am Vet Med Assoc.* 2008;238:1441-1451.

33. Feldman EC. Evaluation of twice-daily lower-dose trilostane treatment administered orally in dogs with naturally occurring hyperadrenocorticism. *J Am Vet Med Assoc.* 2011;238:1441–1451.

34. de Bruin C, Pereira AM, Feelders RA, et al. Coexpression of dopamine and somatostatin receptor subtypes in corticotroph adenomas. *J Clin Endocrinol Metab*. 2009;94:1118-1124.

35. Sato A, Hara Y. Protein expression of somatostatin receptor 2, somatostatin receptor 5 and dopamine D2 receptor in normal pituitary gland and ACTH-secreting pituitary adenoma in dogs. *Res Vet Sci.* 2018;119:61-66.

36. Mangili F, Treppiedi D, Catalano R. A novel mechanism regulating dopamine receptor type 2 signal transduction in pituitary tumoral cells: The role of cAMP/PKA-induced filamin A phosphorylation. *Front Endocrinol.* 2021;11:611752.

37. Treppiedi D, Di Muro G, Mangili F. Filamin A is required for somatostatin receptor type 5 expression and pasireotide-mediated signaling in pituitary corticotroph tumor cells. *Mol Cell Endocrinol.* 2021;524: 111159.

38. Castillo VA, Gomez NV, Lalia JC, et al. Cushing's disease in dogs: cabergoline treatment. *Res Vet Sci.* 2008;85:26-34.

Chapter 2

General introduction

Spontaneous hypercortisolism (HC) manifests through persistent exposure to elevated concentrations of glucocorticoids, leading to both physical and biochemical changes. Approximately 80-85% of hypercortisolism cases in dogs are ACTH-dependent, primarily caused by excessive ACTH secretion from a pituitary corticotroph adenoma. The remaining cases in canine hypercortisolism are ACTH-independent, stemming, in most cases, from the overproduction of glucocorticoids by either benign or malignant adrenocortical tumors.[1]

Morphology and regulation of the canine pituitary gland

The pituitary gland is the core element of the endocrine system. It controls fundamental processes such as metabolism, reproduction, growth, and stress response. Structurally, the pituitary gland consists of three functional units: the anterior lobe (AL, comprising pars distalis and pars tuberalis), the intermediate lobe (IL, known as pars intermedia or PI), and the posterior lobe (PL, forming pars nervosa). The adenohypophysis encompasses both the AL and IL, while the neurohypophysis is constituted by the PL (Figure 1).[2]

In the pars distalis, there are five distinct types of hormone-secreting cells. Corticotroph cells within this region synthesize pro-opiomelanocortin (POMC), giving rise to ACTH. Gonadotroph cells release LH and FSH, somatotroph cells produce growth hormone, while tyrotroph and lactotroph cells are responsible for secreting TSH and prolactin, respectively (Figure 1).[2]

POMC synthesis also occurs in cells of the pars intermedia (PI), where two POMC-producing cell types have been identified. One type, resembling corticotroph cells of the anterior lobe (B cells), reacts with anti-ACTH in immunohistochemical staining. In the other type (A cells), ACTH is cleaved into ACTH1-14 (a precursor of α-melanocyte stimulating hormone (α-MSH)) and corticotropin-like intermediate-lobe peptide (CLIP or ACTH18-39) (Figure 1).[2]

Pituitary secretion and hormone release are regulated by the hypothalamus, which secretes various releasing and inhibiting factors. Among these, dopamine, acting on the specific dopamine D2 receptor, plays a vital role in inhibiting prolactin secretion from lactotroph cells in the pars distalis and ACTH secretion from B cells in the pars intermedia (Figure 1).[2]

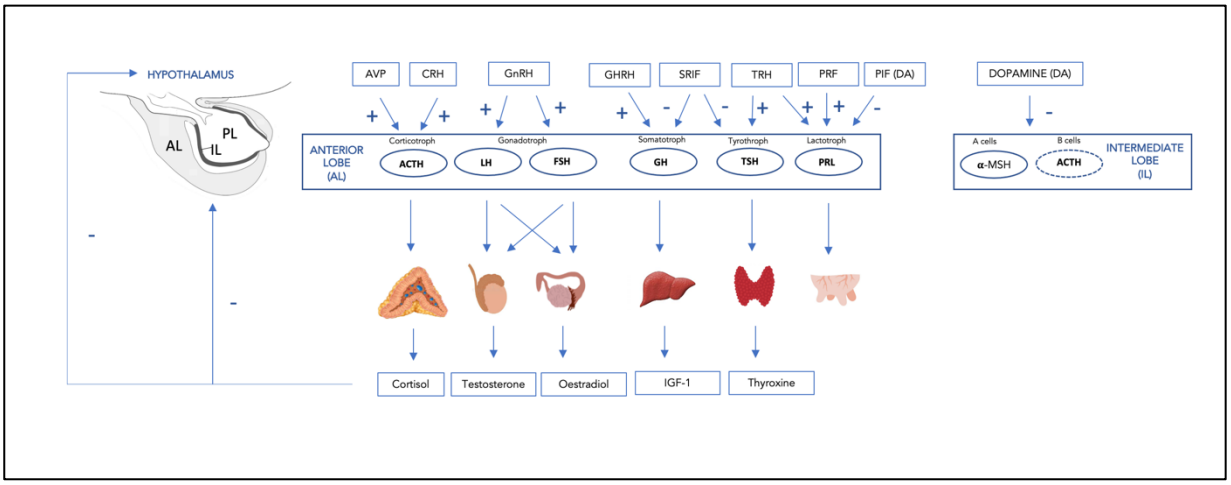

Figure 1. Meij BP, Kooistra HS, Rijneberk AD. Hypothalamus-pituitary system. In: Rijnberk AD, Kooistra HS eds. Clinical Endocrinology of dogs and cats. 2th ed. Hannover, GE:Schlutersche; 2010:13-44.

Pituitary-dependent hypercortisolism (PDH)

Tumors arising from various cell types can develop in the pituitary gland. In humans, the predominant type of pituitary tumors is lactotroph tumors, which secrete prolactin. Conversely, in dogs, the majority of pituitary tumors are corticotroph tumors that produce ACTH.

The ACTH-secreting tumors account for 80-85% of naturally occurring hypercortisolism cases in dogs, with a prevalence of 1 in 500 dogs.[3,4] The heightened secretion of ACTH by the pituitary tumor leads to an increased release of cortisol from the adrenal cortex, resulting in a hypercortisolemic state. This state manifests with the characteristic symptoms associated with Cushing syndrome.[5]

The pituitary lesions responsible for excessive ACTH production encompass a spectrum, ranging from small clusters of hyperplastic corticotrophs to adenomas and sizable pituitary tumors.[6] These tumors can originate from either the AL or the PI. Both lobes contain cells capable of synthesizing POMC, albeit with distinct posttranslational processing.[6] Approximately 20-25% of cases involve a tumor in the PI. This is noteworthy not only due to the tendency for PI tumors to be larger than those in the AL but also because of the divergent hypothalamic control of hormone synthesis in the two lobes.[7-9] This disparity holds clinical significance in the realms of diagnosis and potential alternative medical treatments targeting the pituitary.

Clinical manifestations of hypercortisolism in dogs

Spontaneous hypercortisolism is commonly diagnosed in middle-aged and elderly dogs.[5]

Pituitary-dependent hypercortisolism tends to affect smaller dogs more frequently, with approximately 75% of PDH dogs weighing less than 20 kg, while over 50% of adrenal-dependent hypercortisolism (ADH) subjects weighing more than 20 kg. A certain breed predisposition has been noted in Poodles, Dachshunds, Bichon Frises, Schnauzers, and Fox Terriers.[10-12] No gender predisposition has been proven.[5]

The clinical signs of HC result from the combined effects of cortisol, including gluconeogenic, immunosuppressive, anti-inflammatory, proteo-catabolic, and lipolytic actions.[5]

The clinical picture associated with HC can be highly variable, with some subjects presenting numerous symptoms, while others may be paucisymptomatic. Common historical data include polyuria and polydipsia, polyphagia, abdominal enlargement, alopecia, panting, and muscle atrophy.[5] A less common finding is calcinosis cutis, the dystrophic deposition of calcium salts in the dermis, epidermis, or subcutaneous tissue, which may affect the temporal region, dorsal midline, neck, and abdomen. The pathogenetic mechanism is not entirely clear, but hypercortisolism is hypothesized to increase gluconeogenesis activity and protein catabolism in collagen fibrils, resulting in the formation of a matrix that attracts and binds calcium ions.[5] Even rarer clinical signs include severe muscle stiffness (SMS). This disorder is characterized by persistent bilateral muscle contraction of the thoracic legs, pelvic legs, or all 4 legs (Figure 2). Dogs diagnosed with both HC and stiffness-related muscle syndrome (SMS) typically exhibit the usual clinical signs of HC. However, instead of manifesting muscle weakness, these dogs experience nonpainful SMS, resulting in a bilateral extremely stiff and stilted gait. Even in a prone position, affected dogs display severe and persistent extensor rigidity. Clinically, this combination of SMS in dogs with HC is referred to as "Cushing's myotonia".[13-18] Electromyography results from dogs presenting both HC and SMS reveal "myotonic," bizarre, and high-frequency discharges. Muscle histopathology in dogs with typical HC and weakness involves Type II muscle fiber atrophy, while those with concurrent HC and SMS exhibit fiber size variation, focal necrosis, fiber splitting, subsarcolemmal aggregates, and fatty infiltration. Some of these dogs show evidence of

demyelination on nerve conduction studies, indicating a chronic neuropathy.[5] To date, fewer than 20 dogs with HC and SMS have been documented. In addition to addressing HC, some of these dogs received medications (such as L-carnitine, phenytoin, methocarbamol, or diazepam) to alleviate SMS, but none of the treatments successfully resolved the condition.[13-18] The pathogenesis, treatment, and both short- and long-term prognosis for this condition remain unclear.
In 10-25% of dogs with PDH, neurological symptoms may develop due to the so-called "macroadenoma syndrome." Compression of surrounding nervous structures can lead to anorexia/loss of appetite, stupor, circling, ataxia, tetraparesis, head pressing, and seizures.[19] A recent study highlighted that dogs with macroadenomas tend to have lower body temperature and heart rate compared to dogs with microadenomas, likely due to the compression exerted by the mass on the hypothalamus.[20]

Figure 2. Eleven years old intact male mixed breed dog with a SMS of the pelvic limbs.

Clinicopathological abnormalities

When hypercortisolism is clinically suspected, conducting a comprehensive assessment, including a complete blood count (CBC), serum biochemistry panel, urinalysis, and blood pressure measurement, can provide additional evidence to bolster the diagnosis. Abnormalities identified in these tests may encompass a stress leukogram, elevated serum alkaline phosphatase (ALP) activity, and reduced urine-specific gravity. While none of these findings are conclusive on their own, they can collectively lend support to the suspicion of hypercortisolism.[5]
Hyperphosphatemia frequently occurs in dogs with HC, accompanied by elevated serum parathyroid hormone (PTH) concentrations, a condition previously termed adrenal secondary hyperparathyroidism.[21-23] Dogs affected by PDH exhibit reduced urinary phosphate excretion and increased urinary calcium excretion compared to dogs without hypercortisolism. In contrast to humans, where hypercortisolism often results in elevated calcium and phosphate urinary excretion leading to hypocalcemia and hypophosphatemia, such disturbances are not commonly observed in hypercortisolemic dogs.[21-23] Maintaining calcium and phosphate homeostasis involves a complex interplay among various hormones, with calcitriol, PTH, and fibroblast growth factor-23 (FGF-23) being key players. In humans, hypercortisolism is well-known to impact vitamin D metabolism, particularly in the development of glucocorticoid-induced osteoporosis, a prevalent secondary cause of osteoporosis.[24-26] However, vitamin D metabolism in hypercortisolemic dogs has been insufficiently explored, likely due to the absence of clinically significant osteoporosis

in these animals. A prior study found no significant differences in 25-hydroxyvitamin D (25-(OH)D), calcitriol, and 24,25-(OH)D concentrations between dogs with PDH and healthy dogs.[23]There is a lack of data on plasma FGF-23 concentrations in both hypercortisolemic dogs and humans. Additionally, the simultaneous evaluation of serum concentration, urinary excretion of calcium and phosphate, and serum PTH concentration has not been investigated in hypercortisolemic dogs to date.

Diagnosis of hypercortisolism

The specific endocrine tests are essential for confirming the suspicion of hypercortisolism and are based on the confirmation of two characteristics: (1) increased cortisol production or (2) decreased sensitivity of the hypothalamic-pituitary-adrenal axis, as a consequence of negative feedback exerted by glucocorticoids.[5] However, no endocrine test has a diagnostic accuracy of 100%. It is therefore important to test only those individuals who present clinical signs, alterations in direct physical examination, or clinicopathological changes compatible with Cushing's syndrome, in order to reduce false negatives and false positives. With the increasing prevalence of the disease, diagnostic tests become more accurate.[28]

Specific endocrine tests are divided into screening tests and differentiation tests. Screening tests confirm or deny the diagnosis of HC. Among these are the urinary cortisol to creatinine ratio (UCCR), the ACTH stimulation test (ACTHst), and the low-dose dexamethasone suppression test (LDDST). Differentiation tests, on the other hand, are useful for identifying the origin of the pathology (PDH vs. ADH). These include the LDDSt, high-dose dexamethasone suppression test (HDDST) on blood or urine and measurement of endogenous ACTH. In addition to hormonal tests, differential diagnostic methods include abdominal ultrasound and advanced imaging diagnostics (computed tomography and magnetic resonance).[5]

Treatment

The primary goals in the management of HC involve addressing the root cause of excessive cortisol production, restoring normocortisolism, alleviating clinical symptoms, minimizing long-term complications and mortality, and improving overall quality of life.[29] Treatment strategies and protocols are contingent on various factors. The specific manifestation of hypercortisolism, whether originating from PDH or ADH, guides the approach. Additionally, the severity of the condition, the presence of hypercortisolism-related complications or concurrent diseases, available therapeutic modalities, treatment efficacy, potential side effects, and the preferences of both the clinician and the client contribute to the optimal therapeutic choice. Considerations such as cost implications and the need for frequent follow-up evaluations are also of pivotal significance.

Currently, surgical excision of the causative tumor stands as the sole intervention capable of eliminating either excessive ACTH or autonomous cortisol production. However, these procedures come with inherent risks, have limited accessibility, and may not be universally suitable for every patient. Pharmacotherapy has become a common strategy for addressing clinical symptoms. While surgical intervention is frequently employed for dogs with ADH, most PDH cases are managed through medical therapies, utilizing agents that inhibit adrenocortical hormone synthesis, such as trilostane, or induce partial or complete necrosis of the adrenal cortices, such as mitotane. Both trilostane and mitotane, however, are associated with significant adverse effects. Notably, both medications contribute to decreased plasma cortisol levels and increased ACTH secretion, potentially promoting pituitary tumor growth.[30]

Exploration of novel medical treatments aimed at modulating both adrenal and pituitary function is underway in both human and canine subjects. It is crucial to recognize that not all dogs with spontaneous hypercortisolism, especially those with PDH, require immediate therapeutic intervention. Dogs showing no or minimal clinical symptoms despite abnormal biochemical and endocrine profiles consistent with hypercortisolism should not be subjected to treatment.

Trilostane

Trilostane, a synthetic steroid analog, is the treatment of choice for the medical management of HC. Its mechanism of action involves competitive inhibition of the steroidogenic enzyme 3β-hydroxysteroid dehydrogenase (3βHSD), a key player in the biosynthesis of all adrenocortical hormones. This inhibition disrupts the production of cortisol and aldosterone, leading to a cascade of hormonal responses, including a surge in ACTH levels due to cortisol suppression. This results in the loss of negative feedback and a compensatory increase in plasma renin activity due to reduced aldosterone production.[31]

Since 1998, trilostane has been approved for the medical treatment of both PDH and ADH. However, most investigations into trilostane utilization have focused on canines affected by PDH.[5]

Ensuring the effective management of HC with trilostane necessitates regular and frequent monitoring. Over the past decade, substantial efforts have been dedicated to determining the optimal approach for monitoring trilostane therapy. Irrespective of the chosen method, the evaluation of clinical signs is the initial step.[32-28]

Among the commonly employed monitoring methods, the ACTHst is frequently utilized. This test assesses the adrenal glands' ability to secrete cortisol in response to stimulation and is considered an indicator of cortisol reserve. Despite its widespread use, the ACTHst has not been validated as a definitive monitoring tool for trilostane therapy. Concerns arise due to potential result variability based on test timing and its alignment with clinical control. Additionally, the availability of tetracosactide varies across different countries.

An alternative method recently proposed involves measuring pre-trilostane administration cortisol (prepill) and correlating it with reported clinical signs by pet owners.[32] This approach suggests that cortisol concentration measured after the effects of trilostane have diminished could provide objective insights into optimal dosage and frequency for the dog. While the prepill appears to exhibit the strongest correlation with clinical assessment among various monitoring tests investigated, it still has several limitations. Currently, there is no consensus on whether it serves as the gold standard for monitoring trilostane treatment.

Previous research indicates that trilostane shows an effectiveness range of 67% to 100% in resolving diverse signs of hypercortisolism over a period of 3 to 6 months in both PDH and ADH.[39-47] Common clinical manifestations such as polyuria/polydipsia, polyphagia, and lethargy tend to gradually improve within the initial months of trilostane treatment, while resolution of dermatological irregularities may require additional months. Typical laboratory anomalies associated with HC also show improvement with trilostane treatment.[5]

Approximately 10-15% of dogs treated with trilostane do not exhibit improvement in the clinical picture and clinicopathological variables.[5] Factors predicting trilostane efficacy have not been evaluated to date. Understanding the likelihood of a dog responding to the drug's action can assist veterinarians and owners in managing PDH treatment, considering the potential expense and rigorous monitoring requirements, especially during the initial months of treatment.

In dogs with PDH, trilostane effectively mitigates the clinical manifestations of glucocorticoid excess, although it does not directly influence the growth of the pituitary tumor. While this may be insignificant for dogs initially presenting

with non-enlarged pituitary glands, characterized by a P/B value of ≤ 0.31 mm/mm^2, the pituitary tumor may exhibit growth over time. It is noteworthy that in healthy dogs, the P/B value has been shown to notably increase after trilostane therapy; however, this phenomenon remains unexplored in the context of dogs with PDH.[19] For this reason, pituitary-targeting drugs have gained attention in the management of PDH in recent years.

Pituitary targeting drugs

Effectively managing PDH involves specifically targeting the underlying pituitary tumor. Dopamine (DA) and somatostatin (SST) have emerged as crucial players due to their inhibitory functions within the pituitary gland, prompting the exploration of drugs targeting this gland.
The focus on pituitary-directed drugs revolves around three primary receptor subtypes: dopamine receptor subtype 2 (DRD2), somatostatin receptor subtype 2 (SSTR2), and somatostatin receptor subtype 5 (SSTR5). In canine corticotroph adenomas, the predominant receptor subtype is SSTR2, with DRD2 and SSTR5 expressed at considerably lower levels.[48,49] It's worth noting that there are differences in the distribution of these receptor subtypes when comparing treatment approaches between dogs and humans. In human corticotroph adenomas, the main receptors are SSTR5 and DRD2. Additionally, in humans, the actin-binding protein filamin A (FLNA) is essential for the expression and signaling of SSTR2, SSTR5, and dopamine receptor 2.[50,51] No information is available about FLNA expression in the canine pituitary gland.
In human healthcare, therapeutic protocols recommend medical interventions for Cushing's disease when surgical intervention is not viable or when the disease persists after transsphenoidal hypophysectomy. Notably, there is no explicit preference among available medical treatment options. A recent systematic review evaluated the efficacy and safety of medical interventions for Cushing's disease in humans, revealing comparable disease control proportions achieved through both cabergoline (27–43%) and pasireotide (25–35%).[52] Due to the limited efficacy observed with these drugs, an innovative strategy in development involves the use of DA/SST chimeras.
As of now, there are no registered pituitary-directed drugs approved for addressing PDH in canines.

Cabergoline

Cabergoline operates as a dopamine agonist by binding to the DRD2 receptor. In correlation with the moderate expression of DRD2 in corticotroph adenomas of canines, the response of canine corticotroph cells to cabergoline was relatively modest when studied in vitro. Nevertheless, in vivo experiments yielded noteworthy results: 43% of dogs with pituitary-dependent hypercortisolism (PDH) exhibited positive responses to cabergoline treatment.[53] These responses were characterized by a reduction in clinical manifestations, diminished pituitary adenoma sizes, and lower urinary corticoid-to-creatinine ratios (UCCRs).

References

1. Galac S, Reusch CE, Kooistra HS, et al. Adrenals. In: Rijnberk AD, Kooistra HS. Eds. Clinical Endocrinology of Dogs and Cats. Schlütersche. 2010;93–154.
2. Meij BP, Kooistra HS, Rijnberk AD. Hypothalamus-pituitary system. In: Rijnberk AD, Kooistra HS eds. Clinical Endocrinology of dogs and cats. 2th ed. Hannover, GE:Schlutersche; 2010:13-44

3. Willeberg P, Priester W. Epidemiological aspects of clinical hyperadrenocorticism in dogs (canine Cushing's syndrome). *J Am Anim Hosp Assoc.* 1982;18:717–723.
4. O'Neill DG, Scudder C, Faire JM, et al. Epidemiology of hyperadrenocorticism among 210,824 dogs attending primary-care veterinary practices in the UK from 2009 to 2014. *J Small Anim Pract.* 2016;57(7):365–73.
5. Behrend EN. Canine hyperadrenocorticism. In: Feldman EC, Nelson RW, Reusch CE, et al., eds. Canine and Feline Endocrinology. 4th ed. St. Louis, MO: Elsevier Saunders; 2015:377-451.
6. Peterson ME, Krieger DT, Drucker WD, et al. Immunocytochemical study of the hypophisis of 25 dogs with pituitary dependent hyperadrenocorticism. *Acta Endocrinol. 1982;*101:15-24.
7. Kooistra HS, Greven SH, Mol JA, et al. Pulsatile secretion of alfa-melanocyte-stimulating hormone (alfa-MSH) by the pars intermedia of the pituitary gland and the differential effects of dexamethasone and haloperidol on the secretion of alfa-MSH and adrenocorticotrophic hormone in dogs. *J Endocrinol.* 1997;152:113-121.
8. Bosje JT, Rijnberk A, Mol JA, et al. Plasma concentrations of ACTH precursors correlate with pituitary size and resistance to dexamethasone in dogs with pituitary-dependent hyperadrenocorticism. *Domest Anim Endocrinol.* 2002;22:201-210.
9. Kemppainem RJ, Sartin JL. Evidence for episodic but not circadian activity in plasma concentrations of adrenocorticotropin, cortisol and tiroxine in dogs *J Endocrinol. 1984;*103:219-226.
10. Ling GV, Stabenfeldt GH, Comer KM, et al. Canine hyperadrenocorticism: pretreatment clinical and laboratory evaluation of 117 cases. *J Am Vet Med Assoc. 1979;*174: 1211-1215.
11. O'Neill DG, Scudder C, Faire JM, et al. Epidemiology of hyperadrenocorticism among 210,824 dogs attending primary-care veterinary practices in the UK from 2009 to 2014. *J Small Anim Pract.* 2016;57(7):365–73.
12. Carotenuto G, Malerba E, Dolfini C. et al. Cushing's syndrome-an epidemiological study based on a canine population of 21,281 dogs. *Open Vet J.* 2019;9: 27– 32.
13. Griffiths IR, Duncan ID. Myotonia in the dog: a report of four cases. *Vet Rec.* 1973;93:184-188.
14. Duncan ID, Griffiths IR, Nash AS. Myotonia in canine Cushing's disease. *Vet Rec.* 1977;100:30-31.
15. Greene CE, Lorenz MD, Munnell JF, et al. Myopathy associated with hyperadrenocorticism in the dog. *J Am Vet Med Assoc.* 1979;174:1310-1315
16. Swinney GR, Foster SF, Church DB, et al. Myotonia associated with hyperadrenocorticism in two dogs. *Aust Vet J.* 1998;76:722-724
17. Nagata N, Yuki M. Long-term outcome of myotonia associated with hyperadrenocorticism in 2 dogs. *Can Vet J.* 2015;56:931-933.
18. Nam S, Kang BT, Song KH, Seo KW. Long-term follow up of refractory myotonia associated with hyperadrenocorticism in a Maltese dog. *J Vet Clin.* 2020;37:273-277.
19. Pérez-Alenza D, Melián C. Hyperadrenocorticism in dogs. In: Ettinger SJ, Feldman EC, Côté E, eds. Texbook of Veterinary Internal Medicine. 8th ed. St. Louis, MO: Elsevier; 2017:1795-1811.
20. Benchekroun G, Desquilbet L, Herrtage ME, et al. Dogs with macroadenomas have lower body temperature and heart rate than dogs with microadenomas. *Vet J.* 2017;227: 42-45.
21. Findling JW, Adams ND, Lemann J, et al. Vitamin D metabolites and parathyroid hormone in Cushing–s syndrome: relationship to calcium and phosphorus homeostasis. *J Clin Endocrinol Metab.* 1982;54:1039-1044.
22. Tebb AJ, Arteaga A, Evans H, Ramsey IK. Canine hyperadrenocorticism: effects of trilostane on parathyroid hormone, calcium and phosphate concentrations. *J Small Anim Pract.* 2005;46:537-542.

23. Fracassi F, Malerba E, Furlanello T, Caldin M. Urinary excretion of calcium and phosphate in dogs with pituitary-dependent hypercortisolism: case control study in 499 dogs. *Vet Rec.* 2015;117:625.
24. Skversky AL, Kumar J, Abramowitz MK, Kaskel FJ, Melamed ML. Association of glucocorticoid use and low 25-hydroxyvitamin D levels: results from the National Health and nutrition examination survey (NHANES): 2001-2006. *J Clin Endocrinol Metab.* 2011;96:3838-3845.
25. Tirabassi G, Salvio G, Altieri B, et al. Adrenal disorders: is there any role for vitamin D? *Rev Endocr Metab Disord.* 2017;18:355-362.
26. Giustina A, Adler RA, Binkley N, et al. Consensus statement from 2nd international conference on controversies in vitamin D. *Rev Endocr Metab Disord.* 2020;21:89-116
27. Corbee RJ, Tryfonidou MA, Meij BP, Kooistra HS, Hazewinkel HAW. Vitamin D status before and after hypophysectomy in dogs with pituitary-dependent hypercortisolism. *Domest Anim Endocrinol*. 2012;42:43-49.
28. Behrend EN, Kooistra HS, Nelson R, Reusch CE, Scott-Moncrieff JC. Diagnosis of Spontaneous Canine Hyperadrenocorticism: 2012 ACVIM Consensus Statement (Small Animal). *J Vet Intern Med.* 2013;27(6):1292–304.
29. Sanders K, Kooistra HS, Galac S. Treating canine Cushing's syndrome: current options and future prospects. Vet J. 2018;241:42-51.
30. Teshima T, Hara Y, Takekoshi S, et al. "Trilostane-induced inhibition of cortisol secretion results in reduced negative feedback at the hypothalamic–pituitary axis." *Domest Anim Endocrinol*. 2009;36:32-44.
31. Sieber-Ruckstuhl NS, Boretti FS, Wenger M, Maser-Gluth C, Reusch CE. Cortisol, aldosterone, cortisol precursor, androgen and endogenous ACTH concentrations in dogs with pituitary-dependant hyperadrenocorticism treated with trilostane. *Domest Anim Endocrinol.* 2006;31:63-75.
32. Macfarlane L, Parkin T, Ramsey I. Pre-trilostane and three-hour post trilostane cortisol to monitor trilostane therapy in dogs. *Vet Rec.* 2016;179:597-601.
33. Arenas Bermejo C, Pérez Alenza D, García San José P, et al. Laboratory assessment of trilostane treatment in dogs with pituitarydependent hyperadrenocorticism. *J Vet Intern Med.* 2020;34:1413-1422.
34. McGrotty YL, Arteaga A, Knottenbelt CM, et al. Haptoglobin concentrations in dogs undergoing trilostane treatment for hyperadrenocorticism. *Vet Clin Pathol.* 2005;34:255-258.
35. Galac S, Buijtels JJ, Kooistra HS. Urinary corticoid:creatinine ratios in dogs with pituitary-dependent hyperadrenocorticism during trilostane treatment. *J Vet Intern Med.* 2009;23:1214-1219.
36. Arteaga A, Dhand NK, McCann T, et al. Monitoring the response of canine hyperadrenocorticism to trilostane treatment by assessment of acute phase protein concentrations. *J Small Anim Pract.* 2010;51:204-207.
37. Cook AK, Bond KG. Evaluation of the use of baseline cortisol concentration as a monitoring tool for dogs receiving trilostane as a treatment for hyperadrenocorticism. *J Am Vet Med Assoc.* 2010;237: 801-805.
38. Burkhardt WA, Boretti FS, Reusch CE, Sieber-Ruckstuhl NS. Evaluation of baseline cortisol, endogenous ACTH, and cortisol/ACTH ratio to monitor trilostane treatment in dogs with pituitary-dependent hyperadrenocorticism. *J Vet Intern Med.* 2013;27:919-923.
39. Ruckstuhl NS, Nett CS, Reusch CE. Results of clinical examinations, laboratory tests, and ultrasonography in dogs with pituitary-dependent hyperadrenocorticism treated with trilostane. *Am J Vet Res*. 2002;63:506–512.
40. Galac S, Buijtels JJCWM, Mol JA, Kooistra HA. Effects of trilostane treatment on the pituitary-adrenocortical and renin-aldosterone axis in dogs with pituitary-dependent hypercortisolism. *Vet J.* 2008, doi:10.1016/j.tvjl.208.10.007

41. Braddock JA, Church DB, Robertson ID, Watson AD. Trilostane treatment in dogs with pituitary-dependent hyperadrenocorticism. *Aust Vet J.* 2003;81:600–607.
42. Augusto M, Burden A, Neiger R, Ramsey I. A comparison of once and twice daily administration of trilostane to dogs with hyperadrenocorticism. *Tierarztl Prax K H.* 2012;40:415–424.
43. Arenas C, Melian C, Perez-Alenza MD. Evaluation of 2 trilostane protocols for the treatment of canine pituitary-dependent hyperadrenocorticism: Twice daily versus once daily. *J Vet Intern Med.* 2013;27:1478-1485.
44. Cho KD, Kang JH, Chang D, Na KJ, Yang MP. Efficacy of low-and high-dose trilostane treatment in dogs (< 5 kg) with pituitary-dependent hyperadrenocorticism. *J Vet Intern Med.* 2013;27:91–98.
45. Alenza DP, Arenas C, Lopez ML, Melian C. Long-term efficacy of trilostane administered twice daily in dogs with pituitary-dependent hyperadrenocorticism. *J Am Anim Hosp Assoc.* 2006;42:269–276.
46. Vaughan MA, Feldman EC, Hoar BR, Nelson RW. Evaluation of twice-daily, low-dose trilostane treatment administered orally in dogs with naturally occurring hyperadrenocorticism. *J Am Vet Med Assoc.* 2008;238:1441-1451.
47. Feldman EC. Evaluation of twice-daily lower-dose trilostane treatment administered orally in dogs with naturally occurring hyperadrenocorticism. *J Am Vet Med Assoc.* 2011;238:1441–1451.
48. de Bruin C, Pereira AM, Feelders RA, et al. Coexpression of dopamine and somatostatin receptor subtypes in corticotroph adenomas. *J Clin Endocrinol Metab.* 2009;94:1118-1124.
49. Sato A, Hara Y. Protein expression of somatostatin receptor 2, somatostatin receptor 5 and dopamine D2 receptor in normal pituitary gland and ACTH-secreting pituitary adenoma in dogs. *Res Vet Sci.* 2018;119:61-66.
50. Mangili F, Treppiedi D, Catalano R. A novel mechanism regulating dopamine receptor type 2 signal transduction in pituitary tumoral cells: The role of cAMP/PKA-induced filamin A phosphorylation. *Front Endocrinol.* 2021;11:611752.
51. Treppiedi D, Di Muro G, Mangili F. Filamin A is required for somatostatin receptor type 5 expression and pasireotide-mediated signaling in pituitary corticotroph tumor cells. *Mol Cell Endocrinol.* 2021;524: 111159.
52. Simoes Correa Galendi J, Correa Neto ANS, Demetres M, et al. Effectiveness of Medical Treatment of Cushing's Disease: a systematic review and Meta-analysis. *Front Endocrinol.* 2021;12,:732240.
53. Castillo VA, Gomez NV, Lalia JC, et al. Cushing's disease in dogs: cabergoline treatment. *Res Vet Sci.* 2008;85:26-34.

Chapter 3

Clinical features of muscle stiffness in 37 dogs with concurrent naturally occurring hypercortisolism

Stefania Golinelli, Federico Fracassi, Ezio Bianchi, Alan Gomes Pöppl, Diego Daniel Miceli, Leontine Benedicenti, Viviani De Marco, Audrey K. Cook, Laura Espada Castro, Ian Ramsey, Kyoung Won Seo, Carlo Cantile, Gualtiero Gandini, Sean E. Hulsebosch, Edward C. Feldman

J Vet Intern Med. 2023;37(2):578-585.

Dipartimento di Scienze Mediche Veterinarie,

Scuola di Agraria e Medicina Veterinaria,

Bologna

Abstract

Background: Severe muscle stiffness (SMS) in dogs with hypercortisolism (HC) is uncommon.
Objectives: To evaluate signalment, presentation, treatments, and long-term outcomes of dogs with concurrent HC and SMS.
Animals: Thirty-seven dogs.
Methods: Medical records of dogs with HC and concurrent SMS were recruited from 10 institutions. Clinical information, test results, therapeutic responses, and survival times were reviewed.
Results: All 37 dogs with HC and SMS had pituitary-dependent hypercortisolism (PDH); 36/37 weighed <20 kg. Signs and test results were typical of PDH aside from SMS, initially diagnosed in all 4 limbs in 9, pelvic limbs of 22, and thoracic limbs of 6 dogs. Hypercortisolism and SMS were diagnosed together in 3 dogs; HC 1-36 months before SMS in 23; SMS 1-12 months before HC in 11. Mitotane or trilostane, given to control HC in 36/37 dogs, improved or resolved HC signs in 28; SMS did not resolve, remaining static or worsening in 31/36 dogs, mildly improving in 5/19 dogs given additional therapies. Progression of SMS included additional limbs in 10 dogs and the masticatory muscles of 2. The median survival time from diagnosis of SMS was 965 days (range, 8-1188).
Conclusions and Clinical Importance: Concurrent SMS and HC is uncommon, possibly affecting only dogs with PDH. Development of SMS might occur before or after diagnosis of HC. Apart from SMS, the clinical picture and survival time of these dogs seem indistinguishable from those of dogs with HC in general. However, while muscle weakness usually resolves with HC treatment SMS does not.

Introduction

Naturally occurring and iatrogenic hypercortisolism (HC) are common disorders in dogs, causing clinical signs that include polyuria and polydipsia (PU/PD), polyphagia, thin skin, excess panting, bilaterally symmetrical truncal hair loss, and muscle weakness. Muscle weakness, secondary to chronic glucocorticoid excess, likely contributes to the "pot belly" appearance and exercise intolerance noted frequently in dogs with HC.[1] Severe muscle stiffness (SMS) is rare in dogs with HC and when present has been characterized by persistent bilateral muscle contraction of the thoracic legs, pelvic legs, or all 4 legs. The dogs reported to have concurrent HC and SMS have had typical clinical signs of HC but rather than muscle weakness they have nonpainful SMS resulting in a bilateral extremely stiff and stilted gait. Even when lying down, affected dogs exhibit severe persistent extensor rigidity. The combination of SMS in dogs with HC, clinically, has been referred to as "Cushing's myotonia."[1-10]

Reported electromyography (EMG) results from dogs with HC and SMS include "myotonic," bizarre, high-frequency discharges.[11,12] Muscle histopathology in dogs with typical HC and weakness includes Type II muscle fiber atrophy[1] while those with concurrent HC and SMS have fiber size variation, focal necrosis, fiber splitting, subsarcolemmal aggregates, and fatty infiltration.[10] Evidence of demyelination in some of these dogs on nerve conduction studies (NCS) is consistent with a chronic neuropathy.[12] To the authors knowledge, fewer than 20 dogs with HC and SMS have been described.[2-7] In addition to treating HC, some of those dogs were administered medications (L-carnitine, phenytoin, methocarbamol, or diazepam) to reduce or eliminate the SMS but no treatment resolved the SMS.[6,7] The pathogenesis, treatment, short- and long-term prognosis of dogs with HC and SMS are unclear.[6,7] The aim of the present study was to evaluate a larger number of dogs with concurrent HC and SMS to allow analysis of signalment, presentation, treatment, and outcome.

Materials and methods

In the interest of providing a geographically broad perspective, at least 1 veterinary colleague from Asia, North America, South America, The United Kingdom, and Europe was invited to submit to 1 author (Stefania Golinelli) case information on dogs with concurrent HC and SMS. In total, 14 colleagues from 10 institutions submitted data on dogs from their personal and institutions' records.

Dogs

For inclusion, dogs must have had at least 3 of the following clinical signs of HC: PU/PD, polyphagia, hair loss, thin skin and excessive panting. Dogs must have had obvious abnormal muscle stiffness of the thoracic limbs, pelvic limbs, or all 4 limbs; no evidence of muscle weakness; and no evidence of any other cause for SMS (ie, tetanus). For inclusion, each dog with naturally occurring HC must have had at least 1 abnormal endocrine screening test result (low-dose dexamethasone suppression test [LDDSt]; ACTH stimulation test [ACTHst], urine corticoid: creatinine ratio [UCCR]). Each dog with iatrogenic HC must have had a history of glucocorticoid administration and abnormally suppressed ACTHst and endogenous ACTH [eACTH] results.[1,13] Discriminationof pituitary-dependent hypercortisolism (PDH) from adrenaldependent hypercortisolism (ADH) was determined using results of abdominal ultrasonography, LDDSt, and eACTH concentrations. Dogs with ADH must have had adrenocortical adenoma or carcinoma on adrenal histology.[1] Dogs were excluded if they had signs or laboratory abnormalities inconsistent with HC (ie, vomiting, diarrhea, poor appetite, anemia, increased serum BUN or creatinine concentration). Data submitted for each dog were to include signalment (age,

breed, sex, neuter status, body weight), history, clinical findings, complete blood counts (CBC), routine serum biochemistries, urinalyses, and endocrine test results at time of HC diagnosis, plus the time sequence of diagnosing HC and SMS. Abnormalities detected via diagnostic imaging were to be included. Presence of concurrent disorders must have been described. If performed, results of electrodiagnostic tests (EMG and NCS), muscle, and nerve biopsies were to be submitted. Therapies employed for either HC or SMS and response to treatments were abstracted. "Progression" of SMS was defined as an inability to walk after being ambulatory at diagnosis or when stiffness was observed in additional limbs or masticatory muscles not involved when SMS was initially diagnosed. When available, cause of death was included.

Data Analysis

Collected data were managed with an electronic spreadsheet (Microsoft Excel) and analyzed using a commercial statistical data analysis software program (Prism7.0a, GraphPad Software, Inc, San Diego, California). The Shapiro-Wilk test was used to assess the normality of continuous data. General and clinical characteristics of dogs with HC and concurrent SMS were summarized using mean and SD, median and range, or absolute and percentage frequencies, as appropriate. A Kaplan-Meier curve was performed to assess survival from the time that SMS was diagnosed. Data were censored if the animal was still alive or lost to follow-up at the end of the study period and survival times were reported as medians (range).

Results

Dogs

Thirty-seven dogs with HC and concurrent SMS met the inclusion criteria; 6 from the Federal University of Rio Grande do Sul (Brazil), 5 from the University of Bologna (Italy), 5 from the University of Parma (Italy), 5 from the University of California (USA), 4 from the University of Buenos Aires (Argentina), 3 from the Naya Especialidades of Sao Paulo (Brazil), 3 from the Texas A&M University (USA), 3 from the University of Pennsylvania (USA), 2 from the University of Glasgow (UK)," and 1 from the Chungnam National University (Korea). The earliest date of concurrent HC and SMS diagnosis in a dog was 1984 and the most recent was 2021, with 14 males (9 intact and 5 neutered) and 23 females (5 intact and 18 neutered) in total. Breeds included 13 mixed-breed dogs, 10 Poodles, 4 Dachshunds, 2 Maltese, and 1 each Pembroke Welsh Corgi, Italian Greyhound, Jack Russel Terrier, Lhasa Apso, Italian Hound, Pinscher, Yorkshire Terrier, and Whippet. The median body weight was 7.7 kg (3.2-21) and the mean age at the time of HC and SMS diagnosis was 10.8 years (±2.6) and 11.5 years (±2.6), respectively.

Clinical signs and test results

Clinical signs of HC included PU/PD (35/37 dogs), dermatologic abnormalities (22/37 dogs), polyphagia (21/37 dogs), abdominal enlargement (17/37 dogs), lethargy (7/37 dogs), and panting (4/37 dogs). Several dogs were described as weak at the time that HC was diagnosed, before they developed SMS. No dog had muscle weakness, and all were described as having abnormally "firm" muscles when SMS was diagnosed. CBCs, serum biochemistries and urinalyses from each dog were consistent with HC. Serum creatine kinase activity results were available at diagnosis in 18 dogs; 13 were above the upper reference range (median 460 IU/L, range, 53-3318). Of the 37 dogs, 19 had concurrent medical conditions (Table

1), none of which were believed to alter the diagnosis of HC or SMS although phenobarbital (given to 1 dog) is recognized to interfere with the diagnosis of HC in some dogs.

TABLE 1 Frequency of concurrent medical diseases

Concurrent medical diseases	Number of dogs (%)
Myxomatous mitral valve degeneration	7/37 (19)
Gallbladder diseases	5/37 (14)
Hypothyroidism	4/37 (11)
Neoplasia	3/37 (8)
Dental disease	2/37 (5)
Osteoarthrosis	2/37 (5)
Diabetes mellitus	2/37 (5)
Demodicosis	1/37 (3)
Otitis externa	1/37 (3)
Herniated disk	1/37 (3)
Seizures	1/37 (3)

Results of LDDSt, ACTHst, and UCCR were consistent with a diagnosis of HC in 22, 16, and 5 of 37 dogs, respectively. Test results from each dog were consistent with naturally occurring PDH; no dog had ADH or iatrogenic HC. Testing to discriminate PDH from ADH included abdominal ultrasonography (34 dogs) and eACTH (6 dogs). Pituitary magnetic resonance imaging (4 dogs) and computed tomography (2 dogs) scans were obtained after PDH had been diagnosed. Pituitary masses (2-12 mm at greatest diameter) were seen in 4 dogs. Results of EMGs from 14 dogs exhibited complex repetitive discharges and occasional myotonic discharges, fibrillation potentials, and positive sharp waves (Figure 1).

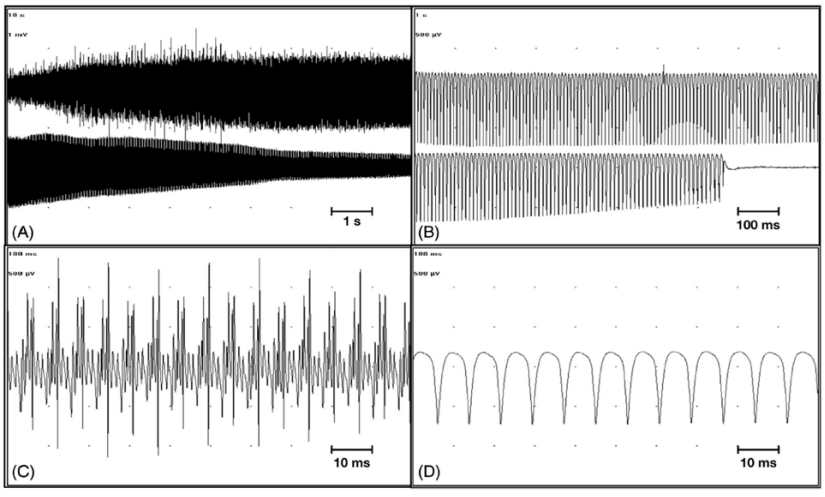

FIGURE 1 High-frequency discharges recorded at electromyography. (A) Myotonic discharges characterized by waxing and waning of amplitudes. (B) Complex repetitive discharges characterized by stable amplitudes and an abrupt end. (C) Spontaneous discharges of single muscle fibers time linked together (complex repetitive discharges). (D) Spontaneous discharges of a single muscle fiber consisting of a train of positive sharp waves

The epaxial and proximal appendicular muscles were more affected than the distal appendicular muscles. Seven dogs underwent NCS. Muscle and nerve biopsies were obtained from 7 dogs. Muscle biopsies demonstrated variation in muscle

fiber size, subsarcolemmal and intermyofibrillar mitochondrial aggregates, moderate fibrosis in 5 out of 7 dogs (Figure 2).

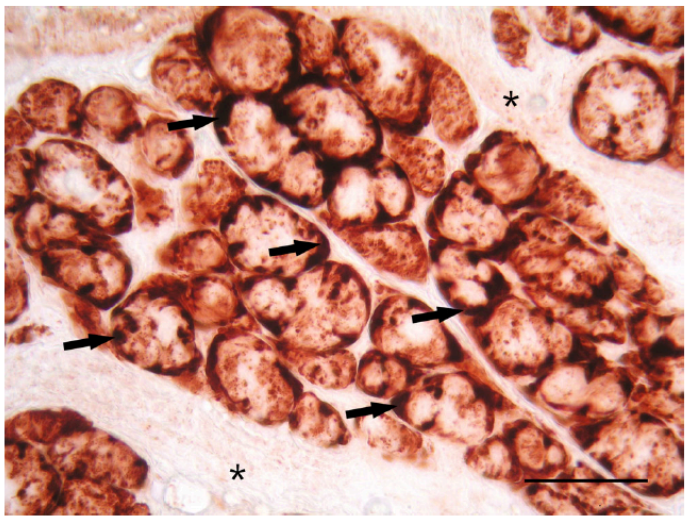

FIGURE 2 Semimembranosus muscle biopsy of 1 dog included in the study. Variation in fiber size, lobular myofibers with accumulation of mitochondria (arrows), and interstitial fibrosis (asterisks). Cryostatic section, cytochrome C oxidase staining, bar = 50 µm

Two dogs had atrophy of type II muscle fibers, 1 of these 2 also had increased muscle fatty deposition. Nerve biopsies demonstrated hypomyelination or demyelination with occasional axonal degeneration in 5 out of 7 dogs (Figure 3).

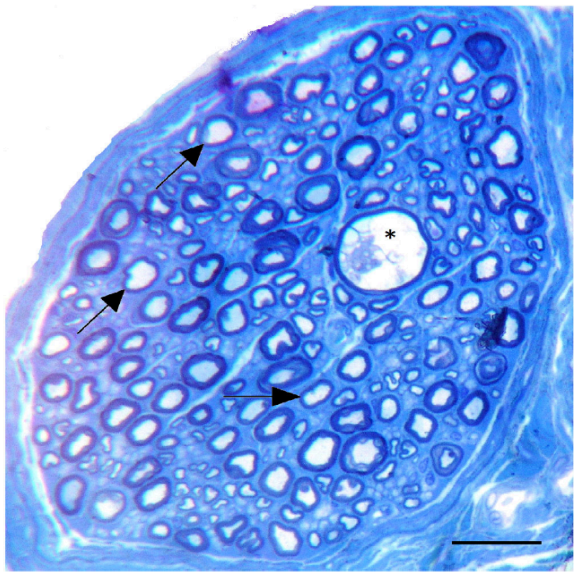

FIGURE 3 Semi-thin section of plastic embedded peroneal nerve biopsy of 1 dog included in the study. Mild loss of nerve fibers, inappropriately thin myelinated fibers (arrows), and occasional axonal degeneration (asterisk). Toluidine blue staining, 500×

Abnormalities were not detected in the histology of 2 dogs.

One dog died 8 days after being diagnosed with HC and SMS before any treatment had been given. Thirty-six dogs were treated for HC, 30 with trilostane (median dose: 1.2 mg/kg; range, 0.5-6.3 mg/kg); 8 received the drug once daily and 22 q12. Eight dogs were treated with mitotane, including 2 dogs after treatment with trilostane did not resolve HC and 1 dog that was also administered melatonin. Clinical signs of HC improved dramatically or resolved in 28/36 treated dogs. Eight

dogs, each treated with trilostane, showed no resolution of HC signs: 5 never responded despite increasing doses, 2 dogs transiently improved and then relapsed despite increasing doses; 1 dog died before completing the first month of treatment. Two dogs initially treated with trilostane without response had resolution of clinical signs after being switched to mitotane. In addition to treatment for HC, 19 of the 36 dogs were given medication for other medical conditions; 5 with ursodeoxycholic acid, 4 with levothyroxine, 3 with clopidogrel, 2 with insulin, 2 with ACE-inhibitors (ie, enalapril and benazepril), 3 with gabapentin, 1 dog each with metronidazole, amlodipine, pregabalin, phenobarbital, and firocoxib. Twenty-three of the 37 dogs were diagnosed with SMS after the initial diagnosis of PDH had been established and treatment begun: 1 dog 1 month later, 14 dogs 2-12 months later, and 8 dogs >1 year later. Nineteen of these 23 dogs had a good response to treatment for HC, 3 failed to demonstrate a response, and 1 was euthanized before completing the first month of trilostane treatment. SMS was diagnosed before PDH in 11 dogs: in 2 dogs 2 months before diagnosis of HC, in 8 dogs 2-12 months before diagnosis of HC and in 1 dog more than 12 months before diagnosis of HC. Hypercortisolism and SMS were diagnosed at the same time in 3 dogs. When limb stiffness was initially identified, only the pelvic limbs of 22 dogs were involved, only the thoracic limbs of 6 dogs, and all 4 limbs in 9 dogs. No dog had 1 or 3 limbs involved and no dog had only unilateral involvement. Difficulty prehending, chewing, or swallowing was not reported in any dog at the time that SMS was diagnosed. No dog appeared in pain to the owner or veterinarian.

Therapies directed at resolving or improving the SMS were administered to 19 dogs (Table 2).

TABLE 2 Frequency of severe muscle stiffness therapeutic intervention used in different dogs

Therapeutic intervention	Number of dogs (%)
Benzodiazepines	7/37 (19)
Physiotherapy	6/37 (16)
Cyclobenzaprine	3/37 (8)
Acupuncture	2/37 (5)
Mexiletine	2/37 (5)
Nonsteroidal anti-inflammatory drugs	2/37 (5)
Dantrolene	1/37 (3)
Botulin toxin	1/37 (3)
L-carnitine	1/37 (3)
Methocarbamol	1/37 (3)
Gabapentin	1/37 (3)
Cannabinoids	1/37 (3)

Mild improvement was noted in 5 dogs treated with diazepam, mexiletine, physiotherapy, acupuncture and cannabis. Some improvement in SMS, after administration of trilostane was stopped because of iatrogenic hypocortisolism, was observed in 1 dog also being treated with physiotherapy and diazepam. Of 5 dogs

that showed mild muscle improvement, 2 were being treated with trilostane once daily and 3 were being treated with trilostane q12. These 5 dogs then had no further change in SMS; 20 dogs never exhibited improvement or progression in SMS after the initial diagnosis; 11 dogs exhibited SMS progression.

Two of 11 dogs (1 treated with trilostane, 1 treated with mitotane) with progressive SMS developed masticatory muscle involvement that caused difficulty chewing and swallowing. The dog treated with mitotane had also been managed with

physiotherapy, acupuncture, and diazepam. The dog treated with trilostane had also been managed with physiotherapy, cyclobenzaprine, botulinum toxin, and diazepam. This dog was still alive at the time of writing and diazepam seemed to help with masticatory muscles relaxation. The other dog was euthanized because of SMS progression. In total, 14 dogs were euthanized, 7 because of persistent or progressive SMS, 1 because of persistent signs of HC, 6 were because of illnesses unrelated to HC, SMS, or its treatment. Three dogs died of other causes, 14 were alive at the time of writing, and 6 were lost to follow-up. Cause of death or euthanasia was not provided for 9 dogs. The median survival time from the diagnosis of SMS was 965 days (8-1188, Figure 4).

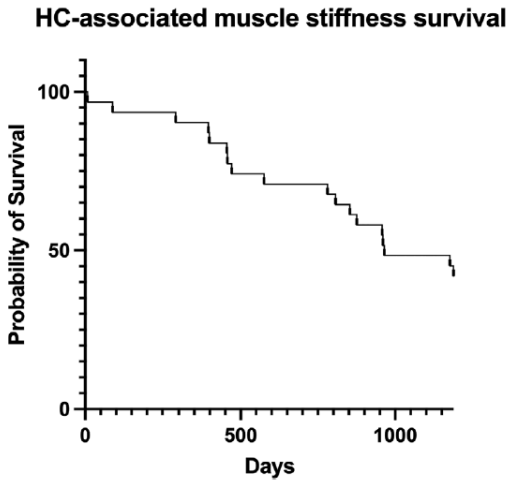

FIGURE 4 Kaplan–Meier curve to show overall survival of dogs with hypercortisolism and concurrent muscle stiffness

Discussion

Breed, sex, age, clinical signs, CBCs, routine serum biochemistries, except for creatinine kinase activities results, and urinalyses at the time of HC diagnosis in the 37 dogs with HC and SMS in this study were similar to those described for dogs with naturally occurring HC but without muscle rigidity.[1,10-18] As a group, the dogs with HC and SMS tended to be small, only 3 dogs weighed >15 kg and just 1 dog weighed >20 kg. The percentage of dogs weighing <20 kg in this study is higher in comparison to that previously reported.1 All 37 dogs included in this study had PDH. Dogs with ADH or iatrogenic HC have excess glucocorticoids, and possibly other adrenal-origin steroids synthesized in a neoplastic adrenal cortex. Dogs with ADH or iatrogenic HC have low-to-undetectable concentrations of pituitary and hypothalamic hormones.[1] Dogs with PDH also have excess glucocorticoids, but the HC is secondary to excess pituitary ACTH. Concentrations of pituitary ACTH and its' prohormones above the reference range at diagnosis, remain above or increase further with adrenaldirected medical treatments.[19,20] Creatinine kinase activity results were above the reference range at diagnosis in the 75% of dogs in which it was measured. In both humans and dogs there are no data available about creatinine kinase concentrations above the reference range in dogs with HC. However, in humans, higher concentrations of CK are described in people with myotonic dystrophy and a mild increase was also reported in a case report of a Chow Chow with congenital myotonia.[21-23] Myotonia, delayed muscle relaxation after voluntary contraction or percussion, occurs in humans, goats, horses, mice, and dogs.[24-41] ACTH and proopiomelanocortin mutations occur in human dystrophic myotonia.[42] In dogs, myotonic signs occur in association with various muscle diseases, as a congenital condition, and in association with HC.[2-7,24-41] Fewer than 20 dogs with HC and concurrent myotonia or SMS are reported.[2-7] In the present study, the case summaries submitted by 14 colleagues from 10 institutions located in widely separated

geographic areas yielded only 37 dogs with concomitant HC and SMS, underscoring the uncommon nature of this combination of conditions. The most common clinical musculoskeletal sign in dogs with HC is nonpainful weakness, recognized by most owners as difficulty rising, abdominal distension, and reduced exercise tolerance.[1] As many as 85% of dogs with HC have been considered weak by the owner and veterinarian,[14] and it is assumed that most of the remaining 15% have a sub-clinical weakness. Steroid-induced Type II muscle atrophy is common in dogs with iatrogenic and naturally occurring HC and since muscle atrophy is a likely component of muscle weakness, it is unlikely that muscle rigidity is a direct consequence of cortisol action. Several possible mechanisms to explain SMS in dogs with HC have been proposed: intracellular potassium concentrations under the reference range, abnormal calcium metabolism, higher glucocorticoid-induced protein catabolism, and alterations in the synthesis of myofibrillar proteins.[3-6] However, the pathogenesis remains unclear. Observing signs of progressive muscle stiffness is subjective for both owners and veterinarians. One report suggested that SMS appeared well after observing other clinical signs of HC.[4] In another report, HC and SMS were diagnosed at about the same time.[6] The time of SMS diagnosis versus time of HC diagnosis in the 37 dogs of this study varied. Twenty-three of 37 dogs (62%) were diagnosed with HC 1 month to 3 years before being diagnosed with SMS, 3 (8%) were diagnosed with HC and SMS at about the same time, and 11 dogs (30%) were diagnosed as having SMS 1 month to 1 year before being diagnosed with HC. Similar to the earlier reports, the limbs involved varied. The majority of dogs (60%) were affected in the pelvic limbs first while 24% had all 4 limbs affected when diagnosed. All dogs diagnosed as having HC and SMS that underwent EGM examinations had myotonic discharges. In these dogs no other abnormalities were identified on muscle biopsies. Despite successful medical management of HC in 28 of 36 treated dogs, only 5 dogs exhibited “mild” SMS improvement, which was followed in each dog by persistent SMS. The SMS persisted or worsened from the time of diagnosis in 31 dogs for which 19 dogs were treated with sodium channel blockers, for example, mexiletine, and muscle
relaxing drugs, for example, methocarbamol, dantrolene, cyclobenzaprine, benzodiazepines, calcium antagonists, and L-carnitine. Such drugs have been efficacious in managing humans with myotonia.[43-45]

One of 36 treated dogs included in this study received botulinum toxin, which was associated with mild muscle relaxation. Botulinum for humans with myotonia has been beneficial.[46,47] Two of 36 dogs treated with physiotherapy exhibited mild muscle relaxation, but there have been no reports of responses to physiotherapy in people with myotonia. The use of cannabinoids and acupuncture resulted in a mild to moderate improvement in 1 dog. Acupuncture has been of some value in humans. Use of cannabinoids in people with myotonia has been associated with some positive results.[48,49] Too few dogs were treated with any single modality to draw conclusions about efficacy.

In addition to worsening limb stiffness, 2/36 of dogs developed progressive difficulty eating and drinking because of masticatory muscle involvement. Inability to eat or drink because of masticatory muscle involvement has not been previously described. One dog did have masticatory muscle abnormalities on EMG in a previous report, but clinical manifestations were not discussed.[3] Masticatory muscle involvement has been described in both human myotonic dystrophy and myotonia congenita.[50,51] Similar to the 2 dogs in this study, masticatory muscle involvement described in humans was preceded by leg muscle involvement. The goal of treating dogs for HC is resolution of clinical signs, achieved by lowering circulating cortisol concentrations.[1] Whether dogs are treated with mitotane or trilostane, owner opinion is recognized as key when determining if a dogs' signs have completely or partially resolved versus dogs with no response. There is no consensus on laboratory testing to aid in monitoring trilostane treatment for dogs with HC and the ACTHst is recognized as ideal for monitoring mitotane treatment.[52-54] Owner observations were a key component in managing the 35 dogs that survived >1 year. In addition, all dogs in this study treated with mitotane were monitored with ACTHst

results while dogs treated with trilostane were monitored with ACTHst results or prepill serum cortisols. The duration of survival after diagnosis of SMS in 3 dogs with HC
in previous studies were 2383, 1902, and 1182 days, respectively.[6,7]
The median survival time from initial diagnosis of SMS in the dogs included in this study was 963 days (range, 8-1188 days). The median survival time for dogs with HC when treated with trilostane or mitotane
has been reported to be 549 to 998 days.[55-58] Despite most dogs having persistent or worsening SMS, owners chose continued care. However, owners of 50% of the dogs in this study ultimately chose euthanasia because of persistent or worsening SMS, highlighting the impact that this condition can have on the dog's and owner's quality of life.
There are several limitations of this study. None of the dogs in this study were treated with hypophysectomy or with drugs targeting the pituitary gland or hypothalamus. Limitations were also associated with the retrospective design of this project and the inclusion of cases from multiple hospitals. Multi-institutional case management was necessary because of the rarity of SMS with HC in a canine population. However, this factor introduced differences in data collection, follow-up, and case treatment based on clinician discretion and varying institutional protocols.

References

1. Behrend EN. Canine hyperadrenocorticism. In: Feldman EC, Nelson RW, Reusch CE, et al., eds. Canine and Feline Endocrinology. 4th ed. St. Louis, MO: Elsevier Saunders; 2015:377-451.
2. Griffiths IR, Duncan ID. Myotonia in the dog: a report of four cases. *Vet Rec*. 1973;93:184-188.
3. Duncan ID, Griffiths IR, Nash AS. Myotonia in canine Cushing's disease. *Vet Rec.* 1977;100:30-31.
4. Greene CE, Lorenz MD, Munnell JF, et al. Myopathy associated with hyperadrenocorticism in the dog. *J Am Vet Med Assoc.* 1979;174: 1310-1315.
5. Swinney GR, Foster SF, Church DB, et al. Myotonia associated with hyperadrenocorticism in two dogs. *Aust Vet J.* 1998;76:722-724.
6. Nagata N, Yuki M. Long-term outcome of myotonia associated with hyperadrenocorticism in 2 dogs. Can Vet J. 2015;56:931-933.
7. Nam S, Kang BT, Song KH, Seo KW. Long-term follow up of refractory myotonia associated with hyperadrenocorticism in a Maltese dog. *J Vet Clin*. 2020;37:273-277.
8. Vite CH. Myotonia and disorders of altered muscle cell membrane excitability. *Vet Clin North Am Small Anim Pract.* 2002;32:169-187.
9. Peterson ME. Hyperadrenocorticism. *Vet Clin North Am Small Anim Pract*. 1984;14:731-749.
10. Braund KG, Dillon AR, Mikeal RL, August JR. Subclinical myopathy associated with hyperadrenocorticism in the dog. *Vet Pathol.* 1980; 17:134-148.
11. Cisneros LE, Palumbo MI, Mortari AC, et al. What is your neurologic diagnosis? Hyperadrenocorticism. J AmVetMed Assoc. 2011;238:1247-1249.
12. Daube JR. Clinical Neurophysiology. 2nd ed. Oxford UK: Oxford University Press; 2002.
13. Behrend EN, Kooistra HS, Nelson R, Reusch CE, Scott-Moncrieff JC. Diagnosis of spontaneous canine hyperadrenocorticism: 2012 ACVIM consensus statement (small animal). *J Vet Intern Med*. 2013; 27:1292-1304.
14. Carotenuto G, Malerba E, Dolfini C, et al. Cushing's syndrome an epidemiological study based on a canine population of 21,281 dogs. *Open Vet J.* 2019;2019(9):27-32.

15. Ling GV, Stabenfeldt GH, Comer KM, et al. Canine hyperadrenocorticism: pretreatment clinical and laboratory evaluation of 117 cases. *J Am Vet Med Assoc*. 1979;1979(174):1211-1215.
16. O'Neill DG, Scudder C, Faire JM, et al. Epidemiology of hyperadrenocorticism among 210,824 dogs attending primary-care veterinary practices in the UK from 2009 to 2014. J Small AnimPract. 2016;57:365-373.
17. Bennaim M, Shiel RE, Mooney CT. Diagnosis of spontaneous hyperadrenocorticism in dogs. Part 1: pathophysiology, aetiology, clinical and clinicopathological features. *Vet J*. 2019;252:105342.
18. Pérez-Alenza D, Melián C. Hyperadrenocorticism in dogs. In: Ettinger SJ, Feldman EC, Côté E, eds. Texbook of Veterinary Internal Medicine. 8th ed. St. Louis, MO: Elsevier; 2017:1795-1811.
19. Witt AL, Neiger R. Adrenocorticotropic hormone levels in dogs with pituitary-dependent hyperadrenocorticism following trilostane therapy. *Vet Rec.* 2004;154:399-400.
20. Sieber-Ruckstuhl NS, Boretti FS, Wenger M, et al. Cortisol, aldosterone, cortisol precursor, androgen and endogenous ACTH concentrations in dogs with pituitary-dependant hyperadrenocorticism treated with trilostane. *Domest Anim Endocrinol*. 2006;31:63-75.
21. Kortz G. Canine myotonia. *Semin Vet Med Surg.* 1989;4:141-145.
22. Brunner HG, Korneluk RG, Coerwinkel-Driessen M, et al. Myotonic dystrophy is closely linked to the gene for muscle-type creatine kinase (CKMM). *Hum Genet*. 1989;81:308-310.
23. Schara U, Schoser BG. Myotonic dystrophies type 1 and 2: a summary on current aspects. *Semin Pediatr Neurol.* 2006;13:71-79.
24. Farrow BRH, Malik R. Hereditary myotonia in the Chow Chow. *J Small Anim Pract.* 1981;22:451-465.
25. Quinn C, Price RS. Myotonia. In: Cucchiara B, Price RS, eds. Decision- Making in Adult Neurology, E-Book. Amsterdam, NL: Elsevier Health Sciences; 2020.
26. Wentink GH, Hartman W, Koeman JP. Three cases of myotonia in a family of chows. *Tijdschr Diergeneeskd.* 1974;99:729-731.
27. Shires PK, Nafe LA, Hulse DA. Myotonia in a Staffordshire terrier. *J Am Vet Med Assoc.* 1983;183(2):229-232.
28. Jones BR. Hereditary myotonia in the Chow Chow. *Vet Ann.* 1984;24: 286-291.
29. Amann JF, Tomlinson J, Hankison JK. Myotonia in a Chow Chow. *J Am Vet Med Assoc.* 1985;187:415-417.
30. Honhold N, Smith DA. Myotonia in the Great Dane. *Vet Rec.* 1986; 119:162.
31. Shores A, Redding RW, Braund KG, Simpson S. Myotonia congenita in a Chow Chow pup. *J Am Vet Med Assoc.* 1986;188:532-533.
32. Hill SL, Shelton GD, Lenehan TM. Myotonia in a cocker spaniel. *J Am Anim Hosp Assoc.* 1995;31:506-509.
33. Rhodes TH, Vite CH, Giger U, et al. A missense mutation in canine C1C-1 causes recessive myotonia congenita in the dog. *FEBS Lett.* 1999;456:54-58.
34. Gracis M, Keith D, Vite CH. Dental and craniofacial findings in eight miniature schnauzer dogs affected by myotonia congenita: preliminary results. *J Vet Dent*. 2000;17:119-127.
35. Bhalerao DP, Rajpurohit Y, Vite CH, Giger U. Detection of a genetic mutationfor myotonia congenita among Miniature Schnauzers and identification of a common carrier ancestor. *Am J Vet Res.* 2002;63: 1443-1447.
36. Finnigan DF, Hanna WJ, Poma R, et al. A novel mutation of theCLCN1 gene associated with myotonia hereditaria in an Australian cattle dog. *J Vet Intern Med.* 2007;21:458-463.
37. Lobetti RG. Myotonia congenita in a Jack Russell terrier. *J S Afr Vet Assoc.* 2009;80:106-107.
38. Quitt PR, Hytönen MK, Matiasek K, et al. Myotonia congenita in a Labrador Retriever with truncated CLCN1. *Neuromuscul Disord.* 2018;28:597-605.

39. de Jesus RD, Damasceno AD, de Araújo CET, et al. Hereditary myotonia in American Bulldog associated with a novel frameshift mutation in the CLCN1 gene. *Neuromuscul Disord.* 2020;30:991-998.
40. de Lahunta A. Myotonia. Veterinary Neuroanatomy and Clinical Neurology. 2nd ed. Philadelphia: Saunders; 1983:86-88.
41. McKerrell RE. Myotonia in man and animals: confusing comparisons. *Equine Vet J*. 1987;19:266-267.
42. Cantara S, Chiofalo F, Ciuoli C, et al. Rare POMC mutation in a patient with myotonic dystrophy type 1 and adrenocorticotropin hyperresponse to corticotropin-releasing hormone. *AACE Clin Case Rep*. 2019;5:132-137.
43. Böhmer T, Rydning A, Solberg HE. Carnitine levels in human serum in health and disease. *Clin Chim Acta.* 1974;57:55-61.
44. Trip J, Drost GG, van Engelen BG, Faber CG. Drug treatment for myotonia. Cochrane Database Syst Rev. 2006.
45. Modoni A, D'Amico A, Primiano G, et al. Long-term safety and usefulness of mexiletine in a large cohort of patients affected by nondystrophic myotonias. *Front Neurol*. 2020;11:300.
46. Bandeira ID, Barretto TL, Santos CV, Lucena R. Botulinum toxin type A in the treatment of facial myotonia in Schwartz-Jampel syndrome. *Muscle Nerve*. 2017;56:10-11.
47. Fernández E, Latasiewicz M, Pelegrin L, et al. A. Botulinum toxin for treating unilateral apraxia of eyelid opening in a patient with congenital myotonia. *Arq Bras Oftalmol.* 2017;80:330-331.
48. Montagnese F, White M, Klein A, et al. Cannabis use in myotonic dystrophy patients in Germany and USA: a pilot survey. *J Neurol.* 2019;266:530-532.
49. Montagnese F, Stahl K, Wenninger S, Schoser B. A role for cannabinoids in the treatment of myotonia? Report of compassionate use in a small cohort of patients. *J Neurol.* 2020;267:415-421.
50. Ödman C, Kiliaridis S. Masticatory muscle activity in myotonic dystrophy patients. *J Oral Rehabil*. 1996;23:5-10.
51. Heatwole CR, Moxley RT. The nondystrophic myotonias. *Neurotherapeutics*. 2007;4:238-251.
52. Arenas Bermejo C, Pérez Alenza D, García San José P, et al. Laboratory assessment of trilostane treatment in dogs with pituitary dependent hyperadrenocorticism. *J Vet Intern Med*. 2020;34:1413-1422.
53. Golinelli S, de Marco V, Leal RO, et al. Comparison of methods to monitor dogs with hypercortisolism treated with trilostane. *J Vet Intern Med.* 2021;35:2616.
54. Neiger R, Hurley K, Ramsey I, et al. Trilostane treatment of 78 dogs with pituitary-dependent hyperadrenocorticism. Vet Rec. 2002;150:799-804.
55. Barker E, Campbell S, Tebb A, et al. A comparison of the survival times of dogs treated with mitotane or trilostane for pituitary-dependent hyperadrenocorticism. *J Vet Intern Med.* 2005;19:810-815.
56. Clemente M, De Andrés PJ, Arenas C, et al. Comparison of nonselective adrenocorticolysis with mitotane or trilostane for the treatment of dogs with pituitary-dependent hyperadrenocorticism. *Vet Rec.* 2007;161:805-809.
57. Fracassi F, Corradini S, Floriano D, et al. Prognostic factors for survival in dogs with pituitary-dependent hypercortisolism treated with trilostane. *Vet Rec*. 2014;176:49.
58. García San José P, Arenas C, Alonso-Miguel D, et al. Survival of dogs with pituitary-dependent hyperadrenocorticism treated twice daily with low doses of trilostane. *Vet Rec*. 2022;191:e1630.

Chapter 4

Calcium and phosphate homeostasis in dogs with newly diagnosed naturally occurring hypercortisolism

Andrea Corsini, Francesco Dondi, Daria G. Serio, Silvia Zamagni,
Stefania Golinelli, Mercedes Fernandez, Federico Fracassi

J Vet Intern Med. 2021;35:1265–1273.

Dipartimento di Scienze Mediche Veterinarie,

Scuola di Agraria e Medicina Veterinaria,

Bologna

Abstract

Background: Hypercortisolism affects calcium and phosphate metabolism in dogs; however, the exact mechanisms are not completely understood.

Objectives: To evaluate circulating concentrations of whole parathormone (wPTH), 25-hydroxyvitamin D (25-(OH)D), calcitriol, and fibroblast growth factor-23 (FGF-23) in dogs with naturally occurring hypercortisolism (NOHC) and healthy dogs, and their association with calcium and phosphate homeostasis.

Animals: Twenty-three client-owned dogs with NOHC, and 12 client or staff-owned healthy dogs.

Methods: Prospective cross-sectional study. The circulating concentrations of total calcium, ionized calcium (iCa), phosphate, wPTH, 25-(OH)D, calcitriol and FGF-23, and the urinary fractional excretion of phosphate (FEP) and calcium (FECa) were compared between dogs with NOHC before treatment and healthy dogs.

Results: Dogs with NOHC had higher mean serum phosphate concentrations (4.81 mg/dL, SD ± 0.71 vs 3.86 mg/dL, SD ± 0.60; P < .001), median FECa (0.43%, range, 0.03-2.44 vs 0.15%, range, 0.06-0.35; P = .005), and median serum wPTH concentrations (54.6 pg/mL, range, 23.7-490 vs 24.6 pg/mL, range, 5.5-56.4; P = .003) as compared to the controls. Circulating concentrations of total calcium, iCa, and calcitriol and the FEP did not differ between groups, whereas the serum 25-(OH)D concentrations were lower in dogs with NOHC as compared to the controls (70.2 pg/mL, SD ± 42.3 vs 106.3 pg/mL, SD ± 35.3; P = .02). The dogs with NOHC had lower plasma FGF-23 concentrations than controls (316.6 pg/mL, range, 120.8-575.6 vs 448.7 pg/mL, range, 244.8-753; P = .03).

Conclusions and Clinical Importance: Urine loss of calcium and hyperphosphatemia could contribute to the adrenal secondary hyperparathyroidism.

Introduction

Naturally occurring hypercortisolism (NOHC) is a common endocrine disease in dogs, characterized by a variety of clinical abnormalities resulting from chronic exposure to excessive concentrations of endogenous glucocorticoids. It is usually caused by an ACTH secreting pituitary adenoma or a functional adrenocortical tumor, with the 2 forms defined as pituitary-dependent hypercortisolism (PDH) and adrenal-dependent hypercortisolism (ADH), respectively.[1] Calcium-containing urolithiasis is a possible, although uncommon, consequence of hypercortisolism, which suggest an influence of this condition on calcium balance.[2] Previous studies have not observed a difference in total and ionized calcium (iCa) concentrations between dogs with NOHC and healthy dogs.[3-6] Hyperphosphatemia occurs frequently in dogs with PDH, together with increased serum parathyroid hormone (PTH) concentrations, a condition previously called adrenal secondary hyperparathyroidism.[3-6] Furthermore, hyperphosphatemia in dogs with newly diagnosed PDH is an independent negative prognostic factor for survival, although the reason behind this finding remains unknown.[7] Dogs with PDH have lower urinary phosphate excretion and higher urinary calcium excretion compared to dogs without hypercortisolism.[5] Hypercortisolism in humans leads to an increase in both calcium and phosphate urinary excretion, occasionally resulting in hypocalcemia and hypophosphatemia.[8]

Calcium and phosphate homeostasis is tightly regulated by a complex interplay between different hormones, with calcitriol, PTH, and fibroblast growth factor-23 (FGF-23) being the most relevant ones.[9] The influence of hypercortisolism on vitamin D metabolism is well-known in humans, mainly as a co-factor in the development of glucocorticoid-induced osteoporosis, the most common secondary cause of osteoporosis in humans.[10-12] Conversely, vitamin D metabolism has been poorly investigated in hypercortisolemic dogs, probably because osteoporosis is not a clinically relevant problem in these animals. In a previous study, 25-hydroxyvitamin D (25-(OH)D), calcitriol, and 24,25-(OH)D concentrations did not differ between dogs with PDH and healthy dogs.[13] No data exist regarding plasma FGF-23 concentrations in either hypercortisolemic dogs or humans. Moreover, the serum concentration, the urinary excretion of calcium and phosphate, and the serum PTH concentration have never been evaluated concurrently in hypercortisolemic dogs.

The aim of this study was to evaluate the regulators of calcium and phosphate homeostasis in dogs with hypercortisolism. The circulating concentrations of calcium, phosphate, PTH, 25-(OH)D, calcitriol, and FGF-23, together with urinary fractional excretion of calcium (FECa) and phosphate (FEP), were compared between dogs with NOHC at the time of diagnosis and healthy dogs. It was hypothesized that the circulating concentrations of PTH, 25-(OH)D, calcitriol, and FGF-23 differed between these samples.

Materials and methods

Study design

A comparative cross-sectional study was designed. Of the dogs presented at the Veterinary Teaching Hospital of the University of Bologna from December 2018 to January 2020, client-owned dogs with newly diagnosed NOHC were prospectively enrolled in the study. A diagnosis of NOHC was made based on clinical signs (eg, polyuria/ polydipsia, polyphagia, dermatological abnormalities and abdominal distension) and laboratory findings, plus a low-dose dexamethasone suppression test (LDDST), an ACTH stimulation test, or both, which were consistent with NOHC. Differentiation between PDH and ADH was achieved based on the evaluation of the endogenous ACTH concentration, LDDST patterns, and diagnostic imaging (abdominal ultrasonography, computed tomography, or both). Age and weightmatched healthy dogs, both client- and hospital staff-owned, presented for routine health checks, were enrolled for

comparison. The dogs were considered healthy on the basis of history, physical examination, and when the results of hematology, a serum chemistry profile, urinalysis, and urine protein to creatinine ratio were within the reference intervals (RIs).

Dogs diagnosed with a concurrent systemic disease (eg, diabetes mellitus, chronic kidney disease, protein-losing enteropathy, other malignant neoplasia, heart failure), dogs previously or currently treated for hypercortisolism, or treated with any formulation of corticosteroids in the month before presentation, dogs receiving diuretics, renin-angiotensin-aldosterone system inhibitors, levothyroxine, phenobarbital, or vitamin D supplementation, and dogs receiving therapeutic diets potentially affecting calcium and phosphate metabolism were excluded from the study. Approval for this study was given by the local Ethical Committee for Animal Testing (ID 1168).

Clinical and clinicopathological data

At the time of inclusion, information regarding concurrent diseases, previous or ongoing medical treatments, and diets were recorded. Blood specimens were collected by standard venipuncture using a blood vacuum system. K3EDTA samples for ACTH evaluation were immediately processed and analyzed within 30 minutes from collection.[14] The urine specimens were collected by cystocentesis or spontaneous voiding. Blood and urine samples were collected at the same time after 12 hours of fasting. The plasma, serum, and urine samples were aliquoted and stored at -80°C for the measurement of PTH, 25-(OH)D, calcitriol, FGF-23, and the urinary excretion of electrolytes.

Venous blood gas analysis, including iCa concentrations, was carried out within 15 minutes from the collection on anaerobically handled heparinized whole blood samples, using a blood gas analyzer (ABL 800 Flex, Radiometer Medical ApS, Brønshøj, Denmark). A complete blood count (CBC) was carried out using an automated hematology system (ADVIA 2120, Siemens Healthcare Diagnostics, Tarrytown, New York). The chemistry profile included serum creatinine, urea, total proteins, albumin, glucose, alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), cholesterol, triglyceride, haptoglobin, total calcium, and phosphorus. Serum chemistry was determined using an automated chemistry analyzer (AU 480, Olympus/Beckman Coulter, Brea, California). Hormone evaluations of cortisol and ACTH were carried out using an automated immunoassay analyzer (Immulite 2000 XPi, Siemens Healthcare Diagnostics, Tarrytown, New York). Urinalysis included urine specific gravity (USG), a dipstick examination, microscopic sediment evaluation, and urine chemistry, in particular the measurement of urinary creatinine (uCr), urine proteins, urine protein to uCr ratio (UPC), and urinary calcium and phosphate.

Urine chemistry was determined using the same automated chemistry analyzer used for the serum chemistry (AU 480, Olympus/ Beckman Coulter, Brea, California); the urinary proteins and uCr were measured using commercially available colorimetric methods (Urinary/CSF Protein OSR6170 and Creatinine OSR6178, Olympus/Beckman Coulter, O'Callaghan's Mills, Ireland). The USG was measured using a hand refractometer (American Optical, Buffalo, New York). Urine dipstick analysis was carried out using a commercially available method (Combur-Test 10 UX, Roche srl, Switzerland) and read by an automated reader (URISYS 1100, Roche srl, Switzerland), the results were additionally confirmed by visual inspection. Microscopic urine sediment examination was carried out at both a low (x100) and a high power field (x400).

The fractional excretion (FE) of calcium (FECa) and phosphate (FEP) were based on a spot urine sample and a contemporaneous serum sample. The FEs were calculated according to a previously reported equation,[15] that is, FEX: (uX x sCr)/(uCr x sX), where uX and sX were the concentrations of the specific analyte in urine and serum, respectively. The results of the FE were reported as percentages, and the reported RIs were the ones defined by the Clinical Pathology

Laboratory of the Institution, which performed the analysis. The FECa was calculated using serum total calcium and urinary calcium concentrations. Serum samples on dry ice were sent to a commercial laboratory (Veterinary Diagnostic Laboratory, Michigan State University, Lansing, Michigan) to evaluate the serum whole parathyroid hormone (wPTH), 25-(OH)D, and calcitriol concentrations, using previously validated radioimmunoassays (RIAs).[16] The calcitriol to 25-(OH)D ratio was calculated. Plasma FGF-23 concentration was assessed using a human-specific FGF-23 ELISA kit (Kainos Laboratories, Tokyo, Japan) previously validated in dogs,[17] after the manufacturer's instructions.

Statistical analysis

The data were analyzed using commercially available software (GraphPad Prism, version 8.0.2, GraphPad Software Inc, San Diego, California). Normality of the continuous data was assessed graphically using the Shapiro-Wilk test. Accordingly, the results were reported as mean and SD, or as median and range (minimum-maximum value). Outliers were detected by the ROUT method (Q = 5%) and removed if considered to be error outliers. Age, total calcium, iCa, phosphate, 25-(OH)D, calcitriol, and FEP were compared between the groups using an unpaired t test. Body weight, FECa, UPC, and the wPTH and FGF-23 concentrations were compared between the groups using the Mann-Whitney test. The categorical variables were compared between groups using the Fisher's exact test. Correlations between the variables in dogs with NOHC were assessed using the Spearman rank test. The Kruskal-Wallis test was carried out to compare dogs with NOHC with increased serum wPTH concentration (above 52.8 pg/mL) with dogs with NOHC with normal serum wPTH concentrations (below or equal to 52.8 pg/mL) and with healthy dogs. Significance was set at $P < .05$.

Results

Study sample

Thirty-three dogs were presented with newly diagnosed NOHC during the study period. Ten dogs were excluded because of absence of blood or urine samples (n = 5), ongoing levothyroxine supplementation (n = 1), myxomatous mitral valve disease with ongoing diuretic treatment (n = 1), chronic kidney disease with severe proteinuria (n = 1), idiopathic epilepsy with ongoing phenobarbital treatment (n = 1), and ongoing topical steroid treatment (eye drops; n = 1). The remaining 23 dogs were enrolled in the NOHC group. Among these dogs, 19 (83%) were diagnosed with PDH and 4 (17%) with ADH. In the same period, 12 healthy dogs were enrolled as a comparison group.

The mean age was 11.1 years (SD ± 2.7) and 10 years (SD ± 1.8) in the hypercortisolemic and the healthy dogs, respectively. Median body weight was 12.7 (range, 3.4-71) and 14 (range, 5-38) kg in the hypercortisolemic and the healthy dogs, respectively. The NOHC group included 12 male (8 neutered) and 11 female (9 spayed) dogs while the healthy dog group included 6 male (1 neutered) and 6 female (2 spayed) dogs. Age, body weight, and sex did not differ between the groups. Breeds in the NOHC group included crossbreeds (n = 12), Maltese and Weimaraners (n = 2), and 1 each of American Bulldog, Beagle, Border Terrier, Cocker Spaniel, Dachshund, Irish Setter, and Newfoundland. The healthy dogs included crossbreeds (n = 4) and 1 each of Bolognese, Border Collie, Dachshund, German Shepard, Italian Shorthaired Segugio, Jack Russel Terrier, Labrador Retriever, and West Highland White Terrier.

Calcium, phosphate, and UPC

The results for the circulating concentrations and the urinary fractional excretions of calcium and phosphate are reported in Table 1.

TABLE 1 Clinicopathological findings regarding calcium and phosphate metabolism in the dogs included in the study

	NOHC dogs (n = 23)		Healthy dogs (n = 12)			
Variables	**n**	**Values**	**n**	**Values**	**RI**	***P* value**
Total calcium (mg/dL)	*23*	10.2 ± 0.7	*12*	10.3 ± 0.69	9.3 to 11	.69
Ionized calcium (mg/dL)	*23*	5.13 (4.65-6.1)	*12*	5.21 (4.85-5.61)	4.97 to 5.65	.26
Phosphate (mg/dL)	*23*	4.81 ± 0.71	*12*	3.86 ± 0.6	2.65 to 5.4	**<.001**
uCa/uCr	*23*	0.06 (0.00-0.29)	*12*	0.02 (0.00-0.03)	0.00 to 0.03	**<.001**
FECa (%)	*23*	0.43 (0.03-2.44)	*12*	0.15 (0.06-0.35)	0.00 to 0.33	**.005**
uP/uCr	*23*	0.97 (0.04-1.72)	*12*	0.61 (0.26-1.68)	0.00 to 0.97	.34
FEP (%)	*23*	12.8 ± 8.9	*12*	17.7 ± 12	2.2 to 27.2	.19
PTH (pg/mL)	*20*	54.6 (23.7-489.6)	*12*	24.6 (5.5-56.4)	4.5 to 52.8	**.003**
25-(OH)D (pg/mL)	*20*	70.2 ± 42.3	*12*	106 ± 35.3	43.7 to 169.5	**.02**
Calcitriol (ng/mL)	*20*	152 ± 44.6	*12*	164 ± 32.1	68.3 to 217.9	.43
Calcitriol to 25-(OH)D ratio	*20*	2.21 (0.83-13.87)	*12*	1.39 (1.03-3.59)	/	.08
FGF-23 (pg/mL)	*22*	316.6 (120.8-575.6)	*12*	448.7 (244.8-753)	/	**.03**

Note: Data are reported as mean ± SD or median and range (minimum-maximum value), based on their distribution. Significance set at *P* value <.05.
Abbreviations: 25-(OH)D, 25-hydroxyvitamin D; FECa, urinary fractional excretion of calcium; FEP, urinary fractional excretion of phosphate; FGF-23, fibroblast growth factor-23; NOHC, natural occurring hypercortisolism; PTH, parathyroid hormone; RI, reference interval; uCa/uCr, urinary calcium to creatinine ratio; uP/uCr, urinary phosphate to creatinine ratio.

Concentrations of total calcium, iCa, and phosphate, and FECa and FEP were available in all dogs. Ionized and total calcium concentrations did not differ between groups (P = .69 and P = .83, respectively). The FECa and the serum phosphate concentrations were higher in the dogs with NOHC as compared with the healthy dogs (P = .005 and P < .001, respectively). Of the dogs with NOHC, 5 (22%) of 23 had a serum phosphate concentration above the upper limit of the RI (4.5-5.4 mg/dL) as compared with none among the healthy dogs. The FEP did not differ between the hypercortisolemic and the healthy dogs (P = .19; Figure 1). The UPC was higher in hypercortisolemic dogs (0.70, range, 0.11-17) as compared with the healthy ones (0.16, range, 0.06-0.31; P < .001).

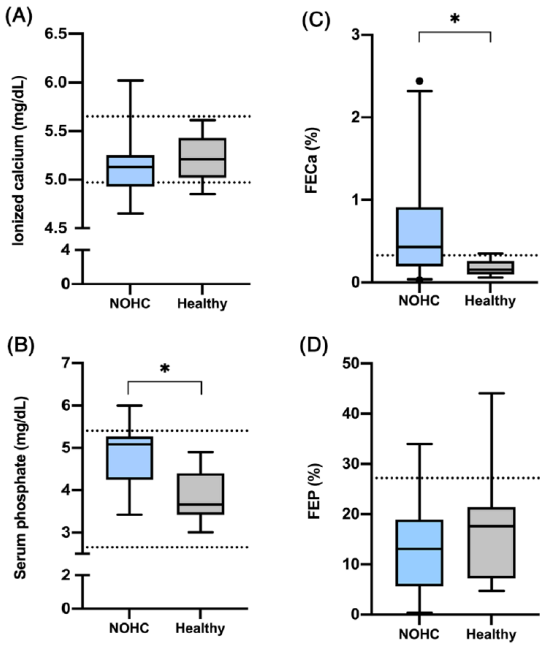

FIGURE 1 Box and whiskers plots comparing (A) ionized calcium concentrations, (B) serum phosphate concentrations, (C) urinary fractional excretion of calcium (FECa), and (D) urinary fractional excretion of phosphate (FEP) in dogs with naturally occurring hypercortisolism (NOHC) and healthy dogs. The boxes represent the interquartile range from the 25th to the 75th percentile. The horizontal bar in each box represents the median value. The whiskers represent the interquartile range from the 2.5th to the 97.5th percentile, with the outliers represented as dots. The dotted lines represent the limits of the reference interval. * $P < .05$

Whole PTH, 25-(OH)D, calcitriol and FGF-23

The results for the calcium and phosphate regulators are reported in Table 1. The concentrations of wPTH, 25-(OH)D, and calcitriol were measured in 20 (86%) of the 23 dogs with NOHC and in all the healthy dogs. Plasma FGF-23 concentrations were measured in all the dogs included in the study. Serum wPTH concentrations were higher in the dogs with NOHC as compared to the healthy dogs (P = .003). Twelve (60%) out of 20 dogs with NOHC and 2 (17%) out of the 12 healthy dogs had serum wPTH concentrations above the upper limit of the RI (4.5-52.8 pg/mL). Serum 25-(OH)D concentrations were lower in the dogs with NOHC as compared to the healthy dogs (P = .02). Five (25%) out of the 20 NOHC dogs and none of the healthy dogs had serum 25-(OH)D concentrations below the lower limit of the RI (43.7-169.5 pg/mL). Serum calcitriol concentrations did not differ between the hypercortisolemic and the healthy dogs (P = .43); only 1 hypercortisolemic dog out of all the dogs included in the study had a value below the lower limit of the RI (68.3-209.5 ng/ mL). Plasma FGF-23 concentrations were lower in the dogs with NOHC as compared to the healthy dogs (P = .03; Figure 2).

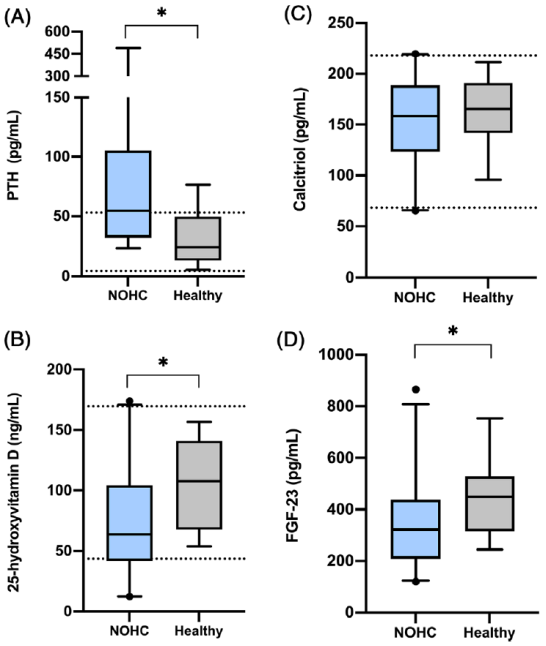

FIGURE 2 Box and whiskers plots comparing serum concentrations of (A) whole parathyroid hormone (wPTH), (B) 25-hydroxyvitamin D, (C) calcitriol, and (D) plasma fibroblast growth factor-23 (FGF-23) concentrations in dogs with naturally occurring hypercortisolism (NOHC) and healthy dogs. The boxes represent the interquartile range from the 25th to the 75th percentile. The horizontal bar in each box represents the median value. The whiskers represent the interquartile range from the 2.5th to the 97.5th percentile, with the outliers represented as dots. The dotted lines represent the limits of the reference interval. * $P < .05$

Calcitriol to 25-(OH)D ratio did not differ between the dogs with NOHC and the healthy dogs (P = .08). When the dogs with NOHC were stratified based on serum wPTH concentrations, those with increased serum wPTH concentrations had a higher median calcitriol to 25-(OH)D ratio (2.92, range, 1.19-13.97) as compared to both the hypercortisolemic dogs with normal wPTH concentrations (1.46, range, 0.83-8.29; P = .02) and the healthy dogs (1.39, range, 1.03-3.59; P = .007).

The calcitriol to 25-(OH)D ratio did not differ between the hypercortisolemic dogs with normal wPTH concentrations and the healthy dogs (P = .98; Figure 3).

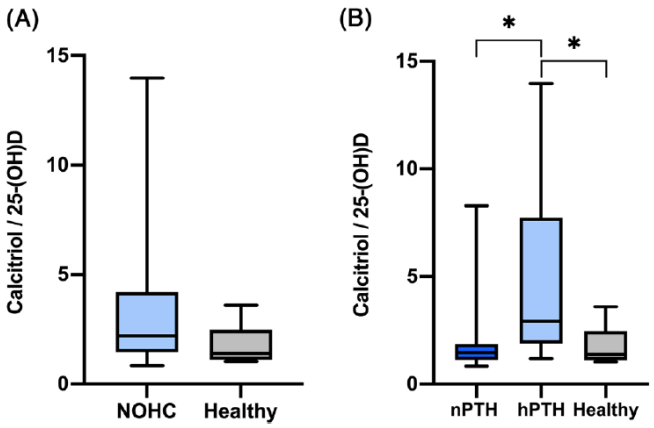

FIGURE 3 Box and whiskers plots comparing the calcitriol to 25-hydroxyvitamin D (25-(OH)D) ratio between (A) dogs with naturally occurring hypercortisolism (NOHC) and healthy dogs and between (B) NOHC dogs with increased serum wPTH concentrations (hPTH), NOHC dogs with normal serum wPTH concentrations (nPTH), and healthy dogs. The boxes represent the interquartile range from the 25th to the 75th percentile. The horizontal bar in each box represents the median value. The whiskers represent the interquartile range from the 2.5th to the 97.5th percentile, with the outliers represented as dots. The dotted lines represent the limits of the reference interval. *$P < .05$

Correlation analysis in the dogs with NOHC

Serum phosphate concentration did not correlate with the FEP nor with the serum concentrations of wPTH, 25(OH)D, calcitriol and plasma FGF-23. The FECa, the FEP, and the iCa, serum wPTH, serum 25-(OH)D, and serum calcitriol concentrations did not correlate with any other parameter. No correlation was found between serum 25-(OH)D concentrations and UPC (r = .36, 95% CI = 0.70 - 0.12; P = .12). The calcitriol to 25-(OH)D ratio showed a moderate positive correlation with the serum wPTH concentration (r = .47, 95% CI = 0.02-0.76; P = .04) and a moderate negative correlation with the plasma FGF-23 concentration (r = .51, 95% CI = 0.78 to 0.08; P = .02; Figure 4). No other correlations were detected.

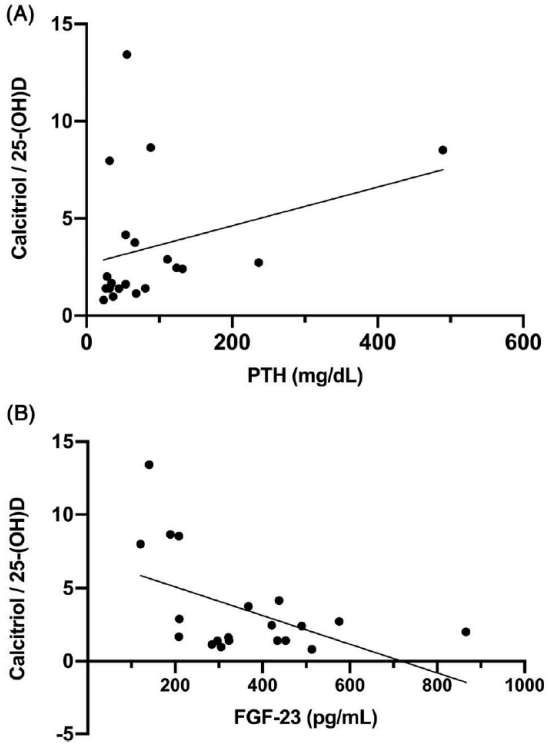

FIGURE 4 Spearman's correlation between (A) the calcitriol to 25-hydroxyvitamin D (25-(OH)D) ratio and serum whole parathyroid hormone (wPTH) concentrations and (B) the calcitriol to 25-(OH)D ratio and plasma fibroblast growth factor 23 (FGF-23) concentrations, in dogs with naturally occurring hypercortisolism

Discussion

This study described the circulating concentrations of the main regulators of calcium and phosphate homeostasis in hypercortisolemic dogs. Dogs with NOHC had higher serum phosphate concentrations, FECa, and serum wPTH concentrations compared to the controls. Whole PTH concentrations were commonly above the upper limit of the RI in the dogs with NOCH. Serum 25-(OH)D concentrations were lower in the dogs with NOHC than in the controls, while calcitriol concentrations did not differ. Also, the dogs with NOHC had lower plasma FGF-23 concentrations.

Hypercortisolism affects calcium homeostasis in different ways.[18,19] In the present study, neither total calcium nor iCa concentrations differed between the groups while dogs with NOHC had increased urinary excretion of calcium. Hypercalciuria is common in hypercortisolemic humans and is described in dogs with PDH.[5,20-22] It is a major risk factor for the development of calcium-containing uroliths, possibly explaining why dogs with NOHC are more likely to develop calcium-containing uroliths as compared to dogs without NOHC,[2] although, in humans with glucocorticoid-dependent nephrolithiasis, multiple factors are involved in kidney urolith formation. [21] Nonetheless, hypocalcemia is rarely reported in humans, and the circulating calcium concentration is largely unaffected in the numerous studies evaluating it in dogs with hypercortisolism.[3-7,13]

The vast majority of studies reports only the total calcium concentrations, and only 2 of them report the iCa concentration.[3,6] Circulating iCa and total calcium concentrations remain within the RI in most of the cases likely because of effective compensation. This compensation could be explained by increased bone resorption, but the present study did not specifically assess the effects of hypercortisolism on bone metabolism. Despite decreased bone mineral density is described in dogs with NOHC compared to healthy dogs, there is no evidence of bone metabolism alterations in dogs with hypercortisolism based on the evaluation of some markers of bone formation and resorption.[6,23] Alternately,

increased intestinal absorption could compensate for increased urinary calcium excretion, but it seems less likely considering that hypercortisolism decreases intestinal calcium absorption in humans.[18] The influence of hypercortisolism on intestinal calcium absorption in dogs was evaluated neither in the present nor in other studies.

Hyperphosphatemia was described in hypercortisolemic dogs in the present study, as had been reported in previous studies.[3,4,6,7,13] The reasons behind this finding are not clear. The urinary excretion of phosphate is decreased in 167 dogs with PDH as compared with healthy and sick control dogs.[5] At least in part, hypophosphaturia could be involved in the development of the hyperphosphatemia described in these dogs. The urinary fractional excretion of phosphate did not differ when dogs with NOHC were compared to healthy dogs in the present study. However, considering that the present results were similar to those reported in the study cited, we believe that the most likely explanation for the findings in the present study is a lack of statistical power because of the markedly lower number of dogs.

In the present study, wPTH concentrations were increased in dogs with NOHC (median value 54.6 pg/mL) as compared to an age and weight-matched sample of healthy dogs. Increased circulating PTH concentrations are reported in dogs with NOHC.[3,6] Mean intact parathyroid hormone (iPTH) concentration, measured using RIA, is approximately 105 pg/mL (exact data not reported, extrapolated from graphs) in a cohort of hypercortisolemic dogs, with 92% among them showing iPTH concentrations above the upper limit of the RI.3 Whole PTH assays are usually regarded as the most precise indicator of actual PTH concentrations since, unlike intact PTH (iPTH) assays, they do not cross-react with large 7-84 PTH fragments. However, the superiority of wPTH assays in routine clinical decision-making remains controversial.[24] Both iPTH and wPTH concentrations, measured using RIAs, are increased in dogs with NOHC as compared to control dogs.

In both these groups, the iPTH is approximately 50% higher than wPTH (median values in dogs with NOHC are 87.2 and 43.74 pg/mL, respectively), with a strong correlation between them. Thus, the measurement

of either provides similar information.[6] Overall, the occurrence of adrenal secondary hyperparathyroidism in hypercortisolemic dogs could be supported by all these studies. Adrenal secondary hyperparathyroidism had been investigated as a cause or as a consequence of calcium and phosphate abnormalities in hypercortisolemic dogs. However, the major function of PTH is to stimulate calcium retention together with phosphate excretion, markedly differing from the calcium and phosphate alterations described in hypercortisolemic dogs. Hypercalciuria occurred in the hypercortisolemic dogs of the present study, supporting the role of abnormal calcium balance in the development of adrenal secondary hyperparathyroidism. Sustained hypercalciuria could stimulate PTH secretion by inducing a mild negative calcium balance despite that the circulating calcium concentrations remained largely unaffected, as reported in this and in previous studies.[5,6,25] Moreover, hyperphosphatemia stimulates the parathyroid

glands to produce PTH independently of changes in serum calcium concentrations.[25] Nonetheless, a correlation between circulating concentrations of calcium and phosphate and PTH was not identified either in the present study or in the previous studies, supporting a multifactorial origin for adrenal secondary hyperparathyroidism.[25]

Cortisol seems to be capable of influencing human and rat parathyroid glands activity both directly, stimulating PTH secretion, and indirectly, decreasing the sensitivity of parathyroid cells to the inhibitory action of calcitriol, thus allowing for an increase in PTH secretion.[26,27] However, hypercalcemia and hypophosphatemia would be expected if these mechanisms contributed primarily to hyperparathyroidism.

Adrenal secondary hyperparathyroidism seems more likely to be a consequence of calcium and phosphate abnormalities. Vitamin D metabolism has been only scarcely investigated in hypercortisolemic dogs. In the present study, the dogs with NOHC had lower serum 25-(OH)D concentrations, whereas the serum calcitriol concentrations did not differ when compared to the healthy dogs. Vitamin D metabolism seems unaffected in a small cohort of 12 dogs with PDH, in which

25-(OH)D, calcitriol, and 24,25-(OH)D do not differ as compared to healthy dogs.[13] Long-term administration of prednisone at 1.5 mg/kg/day slightly decreases serum calcitriol concentrations, but not 25-(OH)D concentrations, in healthy mixed-breed dogs.[28] These findings are similar to what happens in humans undergoing long-term steroid treatment or affected by naturally occurring Cushing's Syndrome, in which circulating concentrations of 25-(OH)D and calcitriol are decreased or unchanged depending on the study.[8,10,29-31] The controversial results reported in the human literature might be because of differences in the nature of the underlying diseases, the dose and duration of the glucocorticoid excess, and the degree of mineral bone disorders.[32] These hypotheses could also apply to the present results. The mechanism of action by which glucocorticoid excess influences vitamin D metabolism in humans is not completely understood; however, it has been proposed that cortisol directly upregulates 24-hydroxylase expression, leading to the increased inactivation of 25-(OH)D and calcitriol.[33,34] Circulating concentrations of 25-(OH)D and calcitriol are decreased in proteinuric non-azotemic dogs in which hypercortisolism was excluded.[35] Urine loss of vitamin D-binding proteins-complexed and albumin-complexed vitamin D metabolites has been suggested as part of a multifactorial explanation, and it could play a role also in hypercortisolemic dogs. However, in the present study no correlation was found between UPC and serum concentrations of 25-(OH)D and calcitriol in dogs with NOHC, despite UPC being higher in dogs with NOHC compared to healthy ones. Despite our study could have failed to demonstrate the impact of proteinuria on circulating 25(OH) concentrations because of inadequate statistical power, it is reasonable to suppose that multiple mechanisms affect circulating 25(OH) concentrations in hypercortisolemic dogs. To additionally assess vitamin D metabolism in the dogs with NOHC, the ratio between calcitriol and 25-(OH)D was calculated. This variable has been proposed to be representative of vitamin D hydroxylation efficiency, possibly improving the understanding of the vitamin D status in the course of the disease.[36] Based on the present results, vitamin D hydroxylation efficiency seemed to be increased in dogs with NOHC with elevated serum wPTH concentrations, as is suggested in the human literature.[8,31] This could partially explain why serum calcitriol concentrations did not differ between the groups in the present study, despite 25-(OH)D being significantly lower in the hypercortisolemic dogs as compared to the healthy dogs. Elevated circulating concentrations of PTH are probably the major factor responsible for the increased 1-α-hydroxylase activity. Nonetheless, the interpretation of the calcitriol to 25-(OH)D ratio remains speculative.

The circulating concentration of FGF-23 had never been evaluated before either in dogs or in humans with hypercortisolism. The major determinant of FGF-23 secretion is the circulating concentration of phosphate, with hyperphosphatemia leading to an increase in plasma FGF-23 concentration.[9,37] In dogs with chronic kidney disease, there is a positive correlation between serum phosphate and plasma FGF-23 concentrations, with FGF-23 markedly increased in more advanced stages of the disease. Serum phosphate concentration is an independent predictor of plasma FGF-23 concentration.[17] Furthermore, phosphate enriched diets induce a mild increase in circulating FGF-23 concentrations in humans, while feeding a phosphate restricted diet is associated with a decrease in circulating FGF-23 concentrations in cats with stable CKD.[38-40] Based on these assumptions, an increased plasma FGF-23 concentration would be expected in hypercortisolemic dogs. In the present study, however, hypercortisolemic dogs showed lower plasma FGF-23 concentrations compared to healthy dogs. No correlation between serum phosphate concentrations and plasma FGF-23 concentrations was found. Notably, even if a difference between hypercortisolemic and healthy dogs exists, its relevance from a clinical standpoint remains unknown. In humans, the clinical consequences of FGF-23-dependant disorders are more commonly reported when an excess of FGF-23 develops, usually because of hereditary genetic diseases or acquired disorders, such as McCune-Albright syndrome or tumor-induced osteomalacia. The clinical phenotype associated with a FGF-23 deficiency had been described only in familial tumoral calcinosis or in experimental FGF-23 knockout mice, and it is characterized by hyperphosphatemia, hypophosphaturia, increased calcitriol concentrations, and ectopic tissue

calcification.[41,42] It could be speculated that, in hypercortisolemic dogs, a decreased plasma FGF-23 concentration plays a role in the development of hyperphosphatemia and hypophosphaturia as described in the previous study.5 However, it is unknown whether the magnitude of the decrease in the plasma FGF-23 concentrations described in the present study could relevantly impact phosphate metabolism. Moreover, the biological activity of FGF-23 depends not only on its circulating

concentrations but also on the tissue expression of the Klotho : FGF receptor complex,[9] which was not assessed in this study. Overall, the interpretation of circulating FGF-23 concentrations in the hypercortisolemic dogs remains unclear.

The present study had some limitations. First, this was an observational comparative cross-sectional study, and the hypothesis proposed remains theoretical. Second, a relatively small number of dogs were included in the study, possibly affecting the statistical power. Thus, the number of dogs included could have been inappropriate for detecting significant differences in some of the variables evaluated, resulting in a type II error. This limitation should be considered when interpreting the lack of difference between groups for some variables described in the present study. For example, a difference in FEP between the groups would have been expected, based on previously published results.[5] Third, the dietary intake of phosphorus, calcium, and 25-(OH)D was not assessed in any of the dogs included in this study, although dogs assuming therapeutic diets were excluded from the study. Thus, even if unlikely, the influence of diet on these results could not be completely ruled out.

References

1. Bennaim M, Shiel RE, Mooney CT. Diagnosis of spontaneous hyperadrenocorticism in dogs. Part 1: pathophysiology, aetiology, clinical and clinicopathological features. *Vet J*. 2019;252:105342.

2. Hess RS, Kass PH, Ward CR. Association between hyperadrenocorticism and development of calcium-containing uroliths in dogs with urolithiasis. *J Am Vet Med Assoc.* 1998;212:1889-1891.

3. Ramsey IK, Tebb A, Harris E, Evans H, Herrtage ME. Hyperparathyroidism in dogs with hyperadrenocorticism. *J Small Anim Pract*. 2005; 46:531-536.

4. Tebb AJ, Arteaga A, Evans H, Ramsey IK. Canine hyperadrenocorticism: effects of trilostane on parathyroid hormone, calcium and phosphate concentrations. *J Small Anim Pract*. 2005;46: 537-542.

5. Fracassi F, Malerba E, Furlanello T, Caldin M. Urinary excretion of calcium and phosphate in dogs with pituitary-dependent hypercortisolism: case control study in 499 dogs. *Vet Rec.* 2015;117:625.

6. Mooney CT, Shiel RE, Sekiya M, et al. A preliminary study of the effect of hyperadrenocorticism on calcium and phosphate concentrations, parathyroid hormone and markers of bone turnover in dogs. *Front Vet Sci.* 2020;7:1-9.

7. Fracassi F, Corradini S, Floriano D, et al. Paper: prognostic factors for survival in dogs with pituitary dependent hypercortisolism treated with trilostane. *Vet Rec*. 2015;176:49.

8. Findling JW, Adams ND, Lemann J, et al. Vitamin D metabolites and parathyroid hormone in Cushing–s syndrome: relationship to calcium and phosphorus homeostasis. *J Clin Endocrinol Metab*. 1982;54:1039- 1044.

9. de Brito Galvao JF, Nagode LA, Schenck PA, Chew DJ. Calcitriol, calcidiol, parathyroid hormone, and fibroblast growth factor-23 interactions in chronic kidney disease. *J Vet Emerg Crit Care.* 2013;23: 134-162.

10. Skversky AL, Kumar J, Abramowitz MK, Kaskel FJ, Melamed ML. Association of glucocorticoid use and low 25-hydroxyvitamin D levels: results from the National Health and nutrition examination survey (NHANES): 2001-2006. *J Clin Endocrinol Metab*. 2011;96: 3838-3845.

11. Tirabassi G, Salvio G, Altieri B, et al. Adrenal disorders: is there any role for vitamin D? *Rev Endocr Metab Disord*. 2017;18:355-362.

12. Giustina A, Adler RA, Binkley N, et al. Consensus statement from 2nd international conference on controversies in vitamin D. Rev Endocr *Metab Disord.* 2020;21:89-116.

13. Corbee RJ, Tryfonidou MA, Meij BP, Kooistra HS, Hazewinkel HAW. Vitamin D status before and after hypophysectomy in dogs with pituitary-dependent hypercortisolism. *Domest Anim Endocrinol*. 2012; 42:43-49.

14. Scott-Moncrieff JC, Koshko MA, Brown JA, Hill K, Refsal KR. Validation of a chemiluminescent enzyme immunometric assay for plasma adrenocorticotropic hormone in the dog. *Vet Clin Pathol.* 2003;32: 180-187.

15. Troìa R, Gruarin M, Grisetti C, et al. Fractional excretion of electrolytes in volume-responsive and intrinsic acute kidney injury in dogs: diagnostic and prognostic implications. *J Vet Intern Med*. 2018;32: 1372-1382.

16. Parker VJ, Harjes LM, Dembek K, Young GS, Chew DJ, Toribio RE. Association of vitamin D metabolites with parathyroid hormone, fibroblast growth factor-23, calcium, and phosphorus in dogs with various stages of chronic kidney disease. *J Vet Intern Med.* 2017;31:791-798.

17. Harjes LM, Parker VJ, Dembek K, et al. Fibroblast growth factor-23 concentration in dogs with chronic kidney disease. *J Vet Intern Med*. 2017;31:784-790.

18. Huybers S, Naber THJ, Bindels RJM, Hoenderop JGJ. Prednisolone-induced Ca2+ malabsorption is caused by diminished expression of the epithelial Ca2+ channel TRPV6. *Am J Physiol*. 2007;292:92-97.

19. Chotiyarnwong P, McCloskey EV. Pathogenesis of glucocorticoidinduced osteoporosis and options for treatment. *Nat Rev Endocrinol*. 2020;16:437-447.

20. Ritz E, Kreusser W, Rambausek M. Effects of glucocorticoids on calcium and phosphate excretion. *Adv Exp Med Biol.* 1984;171:381-397.

21. Faggiano A, Pivonello R, Melis D, et al. Nephrolithiasis in Cushing's disease: prevalence, etiopathogenesis, and modification after disease cure. *J Clin Endocrinol Metab*. 2003;88:2076-2080.

22. Ferrari P, Bianchetti MG, Sansonnens A, Frey FJ. Modulation of renal calcium handling by 11β-hydroxysteroid dehydrogenase type 2. *J Am Soc Nephrol*. 2002;13:2540-2546.

23. Lee D, Lee Y, Choi W, et al. Quantitative CT assessment of bone mineral density in dogs with hyperadrenocorticism. *J Vet Sci*. 2015;16:531-542.

24. Mooney CT, Shiel RE, Fawcett K, Matthews E, Gunn E. A comparison of canine whole and intact parathyroid hormone concentrations as measured by different assays. *J Small Anim Pract.* 2019;60:507-513.

25. Silver J, Levi R. Regulation of PTH synthesis and secretion relevant to the management of secondary hyperparathyroidism in chronic kidney disease. *Kidney Int Suppl*. 2005;67:8-12.

26. Au WYW. Cortisol stimulation of parathyroid hormone secretion by rat parathyroid glands in organ culture. *Science.* 1976;193:1015-1017

27. Karmali R, Farrow S, Hewison M, Barker S, O'Riordan JLH. Effects of 1,25-dihydroxyvitamin D3 and cortisol on bovine and human parathyroid cells. *J Endocrinol.* 1989;123:137-142.

28. Korkor AB, Kuchibotla J, Arrieh M, et al. The effects of chronic prednisone administration on intestinal receptors for 1,25-dihydroxyvitamin D3 in the dog. *Endocrinology.* 1985;117:2267-2273.

29. Seeman E, Kumar R, Hunder GG, Scott M, Heath H 3rd, Riggs BL. Production, degradation, and circulating levels of 1,25-dihydroxyvitamin D in health and in chronic glucocorticoid excess. *J Clin Invest.* 1980;66:664-669.

30. Aloia JF, Roginsky M, Ellis K, et al. Skeletal metabolism and body composition in Cushing's syndrome. *J Clin Endocrinol Metab.* 1974;39:981-985.

31. Kugai N, Shimauchi T, Nagata N, et al. Impaired mineral metabolism in Cushing's syndrome: parathyroid sunction, vitamin D metabolites and osteopenia. *Endocrinol Jpn*. 1986;33:345-352.

32. Muscogiuri G, Altieri B, Penna-Martinez M, Badenhoop K. Focus on vitamin D and the adrenal gland. *Horm Metab Res*. 2015;47:239-246.

33. Akeno N, Matsunuma A, Maeda T, Kawane T, Horiuchi N. Regulation of vitamin D-1α-hydroxylase and 24-hydroxylase expression bydexamethasone in mouse kidney. *J Endocrinol*. 2000;164:339-348.

34. Dhawan P, Christakos S. Novel regulation of 25-hydroxyvitamin D3 24-hydroxylase (24(OH)ase) transcription by glucocorticoids: cooperative effects of the glucocorticoid receptor, C/EBPβ, and the vitamin D receptor in 24(OH)ase transcription. *J Cell Biochem*. 2010;110:1314-1323.

35. Miller MS, Rudinsky AJ, Klamer BG, Chew DJ, Parker VJ. Association between vitamin D metabolites, vitamin D binding protein, and proteinuria in dogs. *J Vet Intern Med.* 2020;34:2468-2477.

36. Pasquali M, Tartaglione L, Rotondi S, et al. Calcitriol/calcifediol ratio: an indicator of vitamin D hydroxylation efficiency? *BBA Clin*. 2015;3:251-256.

37. Takashi Y, Fukumoto S. Phosphate-sensing and regulatory mechanism of FGF23 production. *J Endocrinol Invest.* 2020;43:877-883.

38. Antoniucci DM, Yamashita T, Portale AA. Dietary phosphorus regulates serum fibroblast growth factor-23 concentrations in healthy men. *J Clin Endocrinol Metab.* 2006;91:3144-3149.

39. Vervloet MG, Van Ittersum FJ, Büttler RM, et al. Effects of dietary phosphate and calcium intake on fibroblast growth factor-23. *Clin J Am Soc Nephrol*. 2011;6:383-389.

40. Geddes RF, Elliott J, Syme HM. The effect of feeding a renal diet on plasma fibroblast growth factor 23 concentrations in cats with stable azotemic chronic kidney disease. *J Vet Intern Med*. 2013;27:1354-1361.

41. Liu S, Quarles LD. How fibroblast growth factor 23 works. *J Am Soc Nephrol*. 2007;18:1637-1647.

42. Ito N, Fukumoto S. Congenital hyperphosphatemic conditions caused by the deficient activity of FGF23. *Calcif Tissue Int*. 2021;108:104-115

Chapter 5

Diagnosis of naturally-occurring hypercortisolism by primary care veterinarians: a Western European survey

Miguel F. Carvalho, Rodolfo O. Leal, **Stefania Golinelli**, Federico Fracassi, Carolina Arenas, Maria Pérez-Alenza, Sara Galac, Carmel T. Mooney, Michael Bennaim

Submitted Journal of Veterinary Internal Medicine

Dipartimento di Scienze Mediche Veterinarie,

Scuola di Agraria e Medicina Veterinaria,

Bologna

Abstract

Background: Several diagnostic tests have been described to diagnose naturally-occurring hypercortisolism (HC).

Objectives: To determine how Western European primary care veterinarians (WEPCVs) diagnose canine HC.

Methods: A cross-sectional survey study was conducted, assessing current testing protocols used by WEPCVs for HC screening and differentiation.

Results: 2178 responses from 9 European countries were included. When HC is suspected, 98.7% of respondents indicated performing endocrine testing, while 1.2% rely on treatment trial. Among the former, 59.9% screen a dog for HC without consistent clinical signs but with consistent clinicopathological abnormalities. Of 2150 respondents who perform endocrine testing, 66.6% indicate always using the same initial screening tests regardless of their pre-test suspicion. Among these, the tests most used were the ACTH stimulation test (34.8%), low-dose dexamethasone suppression test (LDDST) (30.4%) or a combination of different tests (25.2%). Where there is no financial constraint, 1419 (66%) respondents always attempt differentiation, using most commonly abdominal ultrasonography (81%) and LDDST (46.1%). Overall, 69.8% of respondents offered referral to an internal medicine or dermatology specialist to ≤20% cases suspected or diagnosed with HC over the previous 5 years.

Conclusions and clinical importance: Testing protocols vary among WEPCVs. Almost 60% of respondents potentially screen for HC in dogs without consistent clinical signs, raising concerns for overdiagnosis. A proportion of WEPCVs never attempt differentiation, which likely affects management strategies and prognosis. Cases are rarely referred to a specialist, reflecting that HC is mainly managed in first-opinion practices. These results suggest that there is room for further education of WEPCVs.

Introduction

Hypercortisolism (HC) is a common endocrine disease in dogs with an estimated prevalence of 0.28% in primary care practice[1]. The most common causes of naturally-occurring HC are overproduction of ACTH by a pituitary tumour (ie, pituitary-dependent HC (PDH)) and autonomous cortisol secretion by a functional adrenal tumour (FAT).[2]

Currently available diagnostic tests for HC exhibit limitations, with frequent false-positive or false-negative results, and no established gold standard test exists.[2] As such, diagnosing HC is a complex process, necessitating a careful interpretation of clinical signs, clinicopathological abnormalities, imaging findings, and results of endocrine tests. Selection of appropriate cases for specific endocrine tests is crucial to maximize diagnostic accuracy.

The diagnostic tests available for investigating HC include the low-dose dexamethasone suppression test (LDDST), the adrenocorticotropic hormone stimulation test (ACTHst), and the urine corticoid-to-creatinine ratio (UCCR). The LDDST takes eight hours to complete and requires the collection of three blood samples. While this test demonstrates a high sensitivity (approximately 90%)[3–6] its specificity remains moderate (approximately 70%) when evaluating dogs with clinically suspected HC[3–6]. The ACTHst is a safe, simple, and time-efficient screening test for HC. Its sensitivity is moderate with, notably, lower values (0 – 63%) reported in dogs with FAT.[3,4,7,8] Despite a widespread belief that this test is highly specific, variables values (59 to 89%) were reported when assessed in dogs with an initial clinical suspicion of HC. [3,7,9–11] The sensitivity of the UCCR is generally agreed to be high with most studies reporting values between 92 and 100%[4,12–16] although one study did show a sensitivity of only 75%.[14] The specificity of this test remains a topic of debate with values ranging from 22% to 85% when evaluated in suspected HC cases.[15–19]

Following a diagnosis of HC, differentiation is recommended to optimize management and prognosticate the disease. The LDDST establishes the cause of HC in about half of the diagnosed cases[6,20], while the high-dose dexamethasone suppression test (HDDST) – which takes another eight hours to complete - provides definitive results in approximately one third of dogs in which the cause remains unclear after a LDDST.[20] Measurement of endogenous ACTH concentration effectively discriminate PDH from FAT[21] and necessitates only a single plasma sample but special handling is required. Imaging modalities, including ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI), can also aid in distinguishing PDH from FAT.

Presumably, the selection of the diagnostic approach for dogs suspected of HC is influenced by individual factors, such as clinical expertise, experience, and personal preferences. One study provided some insights into the diagnostic protocols used by primary care veterinarians in the United Kingdom. In this study, among 191 dogs diagnosed with HC[1], an ACTHst, a LDDST and a UCCR were performed in 95.3%, 33% and 27.8% of cases, respectively. Differentiation was infrequently carried out.

Testing and differentiating protocols used by Western European primary care veterinarians (WEPCVs) remain largely unknown. This study aims to provide an overview of the methods currently employed by WEPCVs to diagnose naturally-occurring HC.

Materials and Methods

The survey methodology for investigating HC testing protocols was structured as follows. Initially, a pilot survey was conducted among 5 board-certified specialists, 9 first-opinion veterinarians and an epidemiologist to identify areas of confusion, unnecessary or missing questions. The final amended questionnaire was translated into five different languages (English, French, Italian, Portuguese, and Spanish) and uploaded into an online survey tool (Google Forms; Alphabet Inc., Mountain View, California). The presented data were voluntarily provided by veterinarians in the full knowledge of the study's objectives. Prior to starting the survey, respondents were required to confirm their understanding that the questionnaire

was exclusively intended for primary care veterinarians and not for specialists. Respondents were required to specify their practice country and only responses from Belgium, France, Ireland, Italy, Luxembourg, Netherlands, Portugal, Spain, or Switzerland were included.

This survey was part of a more comprehensive survey that encompassed inquiries regarding diagnosis and treatment of HC. The data presented in this manuscript only pertains to the diagnosis aspect. The survey (Appendix 1) was divided into three distinct sections: 1) demographics, encompassing inquiries about respondents demographic information including year of graduation, location and type of practice, and number of dogs tested and diagnosed with HC each year; 2) screening protocols , including questions on case selection, tests performed and interpretation of results; 3) differentiation surveying if respondents typically attempt differentiation and, where applicable, which test would be used.

Responses were collected between January and October 2021. To increase the response rate, each questionnaire submitted was entered into a prize draw with a chance to win a € 50 online shopping voucher. The questionnaire was advertised through social network veterinary groups, mailing lists and continuous education events across Western Europe.

One non-native English author (MB) used a generative artificial intelligence tool (GPT 3.5, OpenAI) for rewriting particularly complex sentences in a clearer way, mainly in the discussion section and occasionally in other sections. The sentences generated by the tool were subsequently edited by the author before being incorporated into the manuscript. Furthermore, the draft was reviewed and corrected again by some other coauthors.

Statistical Analysis

The target sample size was 383 determined by identifying the smallest acceptable size with a 5% margin of error and a confidence level of 95%. The number of veterinarians in Western Europe used to calculate the sample size was 97236. This number was obtained adding the estimated number of veterinarians in the nine countries from which data was collected (Belgium, France, Ireland, Italy, Luxembourg, Netherlands, Portugal, Spain and Switzerland) in 2020[22]. Normality of continuous data was assessed by the Shapiro-Wilk test. Descriptive statistics were expressed as numbers and percentages, mean (± standard deviation [SD]) for normally distributed variables and median (range) for non-normally distributed variables. All analyses were performed using commercially available software (IBM SPSS v. 26; IBM Corporation, New York, NY).

Results

Respondents

Overall, 2210 responses were collected. Following review, 32 responses were excluded because respondents indicated practicing outside Europe. Consequently, 2178 responses from 9 European countries were included (Italy [n=1297, 59.6%], France [n= 329, 15.1%], Portugal [n=261, 12.0%], Spain [n=192, 8.8 %], Belgium [n=57, 2.6%], Netherlands [n=18, 0.8%], Republic of Ireland [n=15, 0.7%], Switzerland [n=6, 0.3%] and Luxembourg [n=3, 0.1%]). Respondents had a median experience of 13.0 (0-52) years. Most respondents worked in urban (n=1368; 62.8%) or suburban (n=538; 24.7%) areas, while a smaller proportion practiced in rural (n=266; 12.2%) or other (6; 0.3%) locations. A substantial proportion of respondents (2031; 93.3%) worked exclusively in a small animal practice setting, while 140 (6.4%) worked in mixed practice and 7 (0.003%) indicated working in other practice settings.

Respondents indicated screening HC in 5 (0-450) dogs per year and diagnosing the disease in 3.0 (0-200) dogs per year.

Screening for naturally occurring hypercortisolism

Among all respondents, 1721 (79.0%) indicated always performing hematology, 1384 (63.5%) urinalysis, 1321 (60.7%) abdominal ultrasonography, 1322 (60.7%) electrolytes, 1149 (52.8%) biochemistry, 368 (16.9%) blood pressure measurement, 170 (7.8%) urine culture, and 14 (0.6%) abdominal CT or MRI prior to endocrine testing in dogs suspected of HC. Conversely, a proportion indicated never performing an abdominal CT or MRI (n=1410; 64.7%), blood pressure measurement (n=909; 41.7%), urine culture (n=736; 33.8%), biochemistry (n=719; 33.0%), electrolytes (n=157; 7.2%), abdominal ultrasonography (n=115; 5.3%), urinalysis (n=100; 4.6%), and hematology (n=46; 2.1%) prior to endocrine testing. The remaining respondents indicated doing these tests in a subset of cases (figure 1).

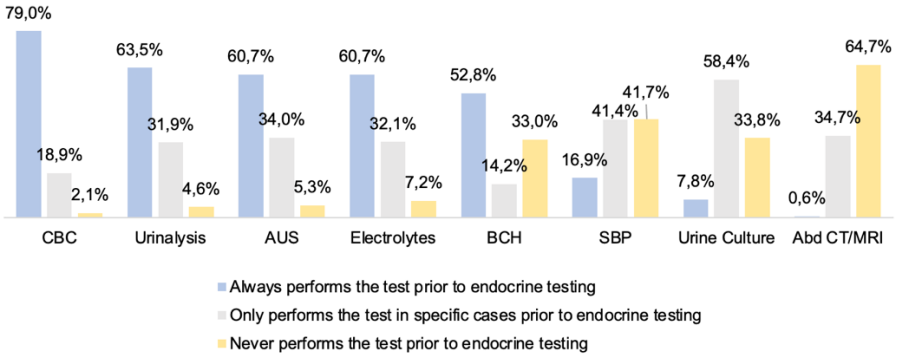

Figure 1. Proportion of respondents who would perform the following tests prior to adrenal function testing in dogs with clinically suspected of HC (n = 2210). CBC: complete blood count; AUS: abdominal ultrasound; BCH: biochemistries; SBP: systemic blood pressure; Abd: abdominal; CT: computer tomography; MRI: magnetic resonance imaging

In order to confirm the diagnosis in a dog clinically suspected of HC, the overwhelming majority of respondents (n=2150; 98.7%) indicated typically performing adrenal function testing, while 29 (1.3%) relied on a treatment trial. Among the former, 1287 (59.9%) respondents indicated they would consider performing screening tests for HC in a dog exhibiting consistent clinicopathological abnormalities but lacking consistent clinical signs, while 1918 (89.2%) indicated they would consider performing screening tests in a dog with consistent clinical signs in the absence of consistent clinicopathological abnormalities. Of 2150 respondents who indicated performing endocrine testing, 1431 (66.6%) indicated always using the same initial screening test or combination of screening tests, while 719 (33.4%) indicated using different screening tests based on their pre-test suspicion. Among the 1431 respondents who indicated always using the same initial screening test, these included the ACTHst (n=498; 34.8%), LDDST (n= 435; 30.4%), UCCR (n= 77; 5.4%), UCCR dexamethasone suppression test (UCCRDST) (n= 42; 2.9%), basal cortisol (n= 18; 1.3%) or a combination of the previous tests (n=36; 25.2%).

Among respondents who indicated always using the same combination of screening tests regardless of their pre-test suspicion, the combinations used include UCCR and LDDST (n= 109; 30.4%), UCCR and ACTHst (n=105; 29.2%), UCCR, ACTHst and LDDST (n=45; 12.5%) and ACTHst and LDDST (n=43; 12.0%). Among the 719 respondents who reported conducting various tests or test combinations based on their pre-test suspicion, 168 (23.4%) consistently performed a LDDST, 111 (15.4%) a UCCR, 106 (14.7%) an ACTHst and 20 (2.8%) a UCCRDST. In contrast, a majority of 437 (60.8%) respondents would never use the UCCRDST, 155 (21.6%) an ACTHst, 87 (12.1%) a LDDST, and 82 (11.3%) a UCCR. Further analysis revealed that among these respondents, 433 (60.2%) would perform for a UCCR, 152

(21.1%) a LDDST, 118 (16.4%) an ACTHst, and 106 (14.7%) a UCCRDST specifically when their pre-test suspicion is low. Conversely, 340 (47.3%) would perform an ACTHst, 312 (43.4%) a LDDST, 156 (21.7%) a UCCRDST, and 93 (12.9%) a UCCR solely where the pre-test suspicion is high (Table 1).

Table 1. Frequency and percentage of various test performed depending on pre-test suspicion (n=719)

	UCCR	UCCRDST	ACTHst	LDDST
	n (%)	n (%)	n (%)	n (%)
NEVER	82 (11.3)	437 (60.8)	155 (21.6)	87 (12.1)
LOW PRE-TEST CLINICAL SUSPICION	433 (60.2)	106 (14.7)	118 (16.4)	152 (21.1)
HIGH PRE-TEST CLINICAL SUSPICION	93 (12.9)	156 (21.7)	340 (47.3)	312 (43.4)
ALWAYS	111 (15.4)	20 (2.8)	106 (14.7)	168 (23.4)

Among 498 respondents who always perform an ACTHst as an initial screening test, 157 (31.5%) and 44 (8.8%) indicated they would not know how to interpret a result within and above reference interval (RI), respectively. Among the remaining respondents, 173 (50.7%) typically find a result within RI sufficient to exclude HC and 418 (92.1%) typically find a result above RI sufficient to confirm HC.

Among 435 respondents who always perform a LDDST as an initial screening test, 140 (32.2%) and 40 (9.2%) indicated they would not know how to interpret a result within and above RI, respectively. Among the remaining respondents, 185 (62.7%) typically find a result within RI sufficient to exclude HC and 358 (90.6%) typically find a result above RI sufficient to confirm HC. Among 77 respondents who always perform a UCCR as an initial screening test, 19 (24.7%) and 8 (10.4%) indicated they would not know how to interpret a result within and above RI, respectively. Among the remaining respondents, 50 (86.2%) find a result within RI sufficient to exclude HC and 17 (24.6%) find a result above RI sufficient to confirm HC. Among 42 respondents who always perform a UCCRDST as an initial screening test, 8 (19%) and 5 (11.9%) indicated they would not know how to interpret a result within and above RI, respectively. Among the remaining respondents, 22 (64.7%) typically find a result within RI sufficient to exclude HC and 35 (94.6%) typically find a result above RI sufficient to confirm HC.

Differentiation between FAT and PDH

Where there was no financial constraint, 1419 (66.0%) and 455 (21.2%) of respondents always and never attempt differentiation, respectively. Among 275 (12.8%) attempting differentiation in specific cases, 52 (18.9%) and 23 (8.4%) undertake it only in larger breeds and smaller breeds, respectively. The 200 (72.7%) remaining respondents indicated deciding to attempt differentiation based on other factors mostly related to the owner. Among 1694 respondents attempting differentiation, 1375 (81.2%) indicated always using abdominal ultrasonography for this purpose, 783 (46.2%) LDDST, 186 (11.0%) head CT or MRI, 179 (10.6%) endogenous ACTH, 147 (8.7%) HDDST, 126 (7.5%) UCCRDST and 111 (6.6 %) abdominal CT or MRI in the absence of financial constraints. Conversely, 26 (1.5%) indicated never performing abdominal ultrasonography, 401 (23.7%) LDDST, 480 (28.3%) head CT or MRI, 977 (57.7%) endogenous ACTH, 858

(50.6%) HDDST, 1085 (64.0%) UCCRDST and 682 (40.3%) abdominal CT or MRI for differentiation. The remaining respondents indicated doing these tests in a subset of cases.

Referral

Out of ten dogs suspected or diagnosed with HC over the last 5 years by each respondent, the median number of dogs for which referral options to an internal medicine or dermatology specialist was offered was 1 (0 – 10). Notably, among 2178 respondents, 1520 (69.8%) indicated providing referral option to ≤ 20% of such cases. Only 178 (8.2%) respondents indicate offering referral to an internal medicine or dermatology specialist to all suspected or confirmed HC cases.

Discussion

The results of this study emphasize the heterogeneity in diagnostic protocols employed by WEPCVs. This variation may be attributed to several factors including the absence of universally established standardized approaches, clinical experience, familiarity with distinct testing methods or the disease itself, and the influence of local academic, clinical training programs and professional organizations.

Our findings suggest that inappropriate selection of cases for endocrine testing is common among WEPCVs, potentially contributing to frequent misdiagnoses. Indeed, a majority of WEPCVs would consider performing screening tests for HC in dogs lacking consistent clinical signs in the presence of consistent clinicopathological abnormalities. Furthermore, key tests essential for building a strong pre-test suspicion, such as biochemistry, abdominal imaging or urinalysis, are consistently performed by only approximately half to two-thirds of WEPCVs before conducting endocrine testing. Given the inherent imperfection of endocrine tests for HC, the consensus among endocrinologists is to conduct these tests only when clinical signs consistent with HC are present[23]. This approach aims to maximize the diagnostic performance of these tests. Furthermore, clinicopathology and imaging are essentials in ruling out alternative differentials for investigated clinical signs. Indeed, false-positive endocrine tests results can occur in the presence of another systemic process.[2] Neglecting to conduct such tests may not only lead to the failure in identifying a concurrent disease that could explain the clinical signs investigated but increases the risk of false positive results and, consequently, erroneous diagnoses.

About two thirds of respondents use the same endocrine test irrespective of their pre-test suspicion while approximately one third use different screening tests based on their pre-test suspicion. This disparity could reflect two different strategies in the investigation of HC. One approach involves testing exclusively in cases with high pre-test suspicion guided by the notion that a positive result in the context of low clinical suspicion might not sufficiently substantiate a diagnosis of HC. An alternative strategy, anecdotally advised, suggests employing tests with a high sensitivity yet lower specificity when the clinical suspicion of HC is low, aiming to effectively exclude the disease.[11] This is likely the rationale of WEPCVs using different tests based on their pre-test suspicion. Indeed, WEPCVs use tests with higher reported sensitivities such as the UCCR particularly in cases with low pre-test suspicion. In contrast, tests with a higher reported specificity such as the ACTHst are favored when there is a high pre-test suspicion. Nonetheless, diagnostic decision-making of WEPCVs extends beyond these strategies. Notably, a majority of WEPCVs would consider testing dogs without clinical signs yet most respondents would use the same initial endocrine test. Furthermore, considering the prevalent acknowledgment of limited interpretational ability for various test results, it is evident that many practitioners might not possess exhaustive knowledge of the intricacies and constraints of each diagnostic test. Other possible explanations include clinical experience and comfort levels with different testing methods, influence of local academic institutions, clinical training programs and professional organizations and economic considerations.

Overall, our findings reveal notable difficulties encountered by WEPCVs in interpreting the screening tests results they commonly employ for the investigation of HC. Approximately one third of respondents who consistently use an ACTHst as a sole initial screening test to investigate HC indicated they would not know how to interpret a result within RI. Among those who perceived themselves as able of interpreting such a result, approximately half would typically exclude HC based on an ACTH-stimulated cortisol concentration within RI. The ACTHst demonstrates only moderate sensitivity with, notably, lower values (0 – 63%) reported in dogs with FAT.[3,4,24–27] Consequently, relying on a post-ACTH cortisol concentration falling within RI for the purpose of ruling out HC is likely to result in the underdiagnosis of cases. Approximately one third of respondents who consistently use a LDDST as a sole initial screening test to investigate HC indicated they would not know how to interpret a result within RI. Among the remaining respondents, about one third would typically find a result within RI insufficient to exclude HC. The LDDST has a consistently reported high sensitivity ranging from 85% to 100%.[3,5,6,14,26,28,29] Thus, a result within RI serves as a strong indicator against HC. Unless there exists a considerably heightened clinical suspicion, it may be unnecessary to pursue further investigation for HC when such a result is obtained. However, the noteworthy proportion of WEPCVs who do not typically deem a result within RI as sufficient evidence to exclude HC might lead to unwarranted follow-up testing. This could potentially result in the misinterpretation of subsequent test results and consequent misdiagnoses. Among those who consistently use the UCCR as a sole initial screening test, approximately a quarter acknowledged uncertainty in interpreting results within RI and most indicated feeling able to interpret a result above RI. Among the latter, approximately one quarter find a result above the RI as adequate to confirm HC. It is widely accepted that the sensitivity of the UCCR is high (92% - 100%)[12–17], although one study reported a sensitivity of only 75%.[14] The specificity of the UCCR remains subject to debate, with values spanning from 22% to 85% within suspected HC cases[15–19]. A notable observation has been the distinction in UCCR use between Dutch academic teams and non-Dutch authors. Dutch teams employ the UCCR as a confirmatory test, while non-Dutch authors often advocate its use for ruling out HC. Historically, the rationale behind this divergence was linked to the use of a radioimmunoassay available solely in the Netherlands. This assay employs anti-cortisol antibodies with minimal cross-reactivity to cortisol metabolites, theoretically yielding the UCCR a higher specificity compared to other assays. However, although assays have not been compared in the same study, recent studies reported similar specificities of the UCCR using more widespread assays, when assessed in an appropriate population[16,19], thus challenging the historical assumptions regarding assay-related variations in UCCR specificity.

Around one third of WEPCVs do not consistently attempt differentiation when there are no financial constraints. Furthermore, approximately one fifth never try to differentiate between PDH and FAT in dogs diagnosed with HC. This likely affects management strategies and the ability to prognosticate the disease[2,23,30], since FAT and PDH have different survival times[31–35] and subsequent distinct surgical approaches[36,37]. Abdominal ultrasonography[38] and LDDST are the tests the most frequently consistently used to differentiate the type of HC. Abdominal ultrasonography is favoured likely due to its widespread availability and ease of use. Additionally, LDDST is likely popular because of the possibility to confirm and differentiate HC in a single test. Although endogenous ACTH, is the most accurate stand-alone biochemical differentiating test[23], it is consistently used by less than 11% of respondents and never used by approximately two thirds of respondents. Endogenous ACTH measurement requires special sample handling. For this reason, other tests easier to perform may be initially favoured before considering eACTH measurement. Additionally, some WEPCVs may be discouraged by the need of special sample handling. Advanced imaging is never used by a significant proportion of WEPCVs. This may reflect the limited availability of these modalities. Advanced imaging is most beneficial when surgery or radiation therapy is being considered or when investigating cases with conflicting results from other differentiating

tests. As a result, their use is limited to specific cases, which could also explain the small proportion of WEPCVs making use of them.

Our results highlight the infrequent practice among WEPCVs to provide owners with the option of specialist evaluation and reveals that that this disease is mainly managed in a primary care setting in Western Europe. Approximately only 8% of these veterinarians consistently extend the opportunity for specialist assessment to cases either suspected or confirmed to have HC. Conversely, approximately 70 % of WEPCVs offered referral to ≤ 20% of such cases. While our study does not delve into the underlying reasons for this observation, several potential explanations could be considered including limited awareness of WEPCVs about the potential benefits of specialist evaluation for such cases, time and resource constraints impacting their ability to facilitate specialist referrals, perceived confidence in their own diagnostic abilities and geographical accessibility. Additionally, some WEPCVs might hesitate to recommend specialist evaluation to owners with financial constraints.

This study encountered several limitations that warrant consideration. A notable limitation arises from the uneven distribution of responses, with a substantial overrepresentation from Italy in comparison to other Western European countries. This skewed distribution could potentially introduce bias in the reported behaviours, favouring Italian veterinary practices. This overrepresentation might be attributed to the larger veterinarian population in Italy[39] or a more effective survey dissemination within the country. Moreover, responses from countries such as the Netherlands and Ireland were comparatively sparse, potentially diminishing the study's ability to fully capture the diversity of practices across Western Europe. The obtained sample exhibited a relatively low proportion of responses from mixed practice veterinarians (less than 6%), implying that the study's findings might be more representative of behaviours within 100 % small animal practices. Recruitment methods could have inadvertently targeted specific subsets of respondents, such as younger veterinarians or veterinarians actively engaged in continuous professional development. The voluntary nature of survey participation introduces the possibility of self-selection bias, where respondents with a heightened interest or awareness in the topic might have been more inclined to participate. Consequently, some behaviours could be overrepresented in the collected data. Although the survey provided valuable quantitative insights, it did not delve into qualitative aspects of respondents' decision-making processes. This absence of in-depth qualitative insights limits our understanding of the underlying motivations and barriers influencing diagnostic choices.

Conclusion

This study provides valuable insights into the varied diagnostic approaches used by WEPCVs in the assessment of canine naturally-occurring HC. A majority of WEPCVs potentially initiating HC screening in dogs lacking consistent clinical signs. Furthermore, a significant proportion of WEPCVs do not consistently perform crucial tests to establish a pre-test suspicion. The study also reveals challenges faced by WEPCVs in interpreting the results of the screening tests they commonly use. Approximately one third of WEPCVs do not consistently attempt to differentiate between the underlying causes of HC, potentially impacting treatment strategies and long-term prognosis. Despite this, referral options to a specialist are rarely offered to animal owners of suspected or diagnosed cases. These findings raise concern for frequent misdiagnosis and suboptimal management of diagnosed cases. These results underscore that there is room for further education of WEPCVs. Heightened awareness about appropriate case selection, limitations of screening tests and the value of specialist input could substantially enhance the overall quality of care provided to these cases.

References

1. O'Neill DG, Scudder C, Faire JM, et al. Epidemiology of hyperadrenocorticism among 210,824 dogs attending primary-care veterinary practices in the UK from 2009 to 2014. *J Small Anim Pract*. 2016;57(7):365–73.

2. Bennaim M, Shiel RE, Mooney CT. Diagnosis of spontaneous hyperadrenocorticism in dogs. Part 2: Adrenal function testing and differentiating tests. *Vet J.* 2019;252:105343.

3. Feldman EC. Comparison of ACTH response and dexamethasone suppression as screening tests in canine hyperadrenocorticism. *J Am Vet Med Assoc*. 1983;182:506–10.

4. Meijer JC, Lubberink AAME, Rijnberk A, et al. Adrenocortical function tests in dogs with hyperfunctioning adrenocortical tumours.. *J Endocrinol.* 1979;80:315–9.

5. Zeugswetter FK, Valencia AC, Glavassevich K, et al. Patterns of the low-dose dexamethasone suppression test in canine hyperadrenocorticism revisited. *Vet Clin Pathol.* 2021;50:62–70.

6. Bennaim M, Shiel RE, Forde C, et al. Evaluation of individual low-dose dexamethasone suppression test patterns in naturally occurring hyperadrenocorticism in dogs. *J Vet Intern Med*. 2018;32:967–77.

7. Peterson ME. Diagnosis of Hyperadrenocorticism in Dogs. Clin Tech Small An P. 2007;22:2–11.

8. Behrend EN. Canine hyperadrenocorticism. In: Feldman EC, Nelson RW, Reusch CE, et al., eds. Canine and Feline Endocrinology. 4th ed. St. Louis, MO: Elsevier Saunders; 2015:377-451.

9. Nivy R, Refsal KR, Ariel E, et al. The interpretive contribution of the baseline serum cortisol concentration of the ACTH stimulation test in the diagnosis of pituitary dependent hyperadrenocorticism in dogs. *J Vet Intern Med.* 2018;32:1897–902.

10. Lanen KV, Sande A. Canine Hypoadrenocorticism: Pathogenesis, Diagnosis, and Treatment. *Top Companion Anim M*. 2014;29:88–95.

11. Gilor C, Graves TK. Interpretation of Laboratory Tests for Canine Cushing's Syndrome. *Top Companion Anim M*. 2011;26:98–108.

12. Stolp R, Rijnberk A, Meijer JC, et al. Urinary corticoids in the diagnosis of canine hyperadrenocorticism. *Res Vet Sci.* 1983;34:141–4.

13. Smiley LE, Peterson ME. Evaluation of a Urine Cortisol:Creatinine Ratio as a Screening Test for Hyperadrenocorticism in Dogs. *J Vet Intern Med.* 1993;7:163–8.

14. Kaplan AJ, Peterson ME, Kemppainen RJ. Effects of disease on the results of diagnostic tests for use in detecting hyperadrenocorticism in dogs. *J Am Vet Med Assoc.* 1995;207:445–51.

15. Jensen AL, Iversen L, Koch J, et al. Evaluation of the urinary Cortisol: creatinine ratio in the diagnosis of hyperadrenocorticism in dogs. *J Small Anim Pr.* 1997;38:99–102.

16. Zeugswetter F, Bydzovsky N, Kampner D, et al. Tailored reference limits for urine corticoid:creatinine ratio in dogs to answer distinct clinical questions. *Vet Rec.* 2010;167:997–1001.

17. Rijnberk A, Wees A van, Mol J. Assessment of two tests for the diagnosis of canine hyperadrenocorticism. *Vet Rec.* 1988;122:178.

18. Feldman EC, Mack RE. Urine cortisol:creatinine ratio as a screening test for hyperadrenocorticism in dogs. *J Am Vet Méd Assoc.* 1992;200:1637–41.

19. Schäfer I, Rehbein S, Holtdirk A, et al. Diagnostic cut-off values for the urinary corticoid:creatinine ratio for the diagnosis of canine Cushing's syndrome using an automated chemiluminescent assay. *Vet Clin Pathol*. 2023.

20. Feldman EC, Nelson RW, Feldman MS. Use of low- and high-dose dexamethasone tests for distinguishing pituitary-dependent from adrenal tumor hyperadrenocorticism in dogs. *J Am Vet Med Assoc.* 1996;209:772–5.

21. Piñeiro MIR, Benchekroun G, Fornel-Thibaud PD, et al. Accuracy of an Adrenocorticotropic Hormone (ACTH) Immunoluminometric Assay for Differentiating ACTH-Dependent from ACTH-Independent Hyperadrenocorticism in Dogs. *J Vet Intern Med.* 2009;23:850–5.

22. FVE's Strategy [Internet]. [cited 2023 Jun 15]. Available from: https://fve.org/cms/wp-content/uploads/FVE_Strategy_2020_R1_Web.pdf

23. Behrend EN, Kooistra HS, Nelson R, et al. Diagnosis of Spontaneous Canine Hyperadrenocorticism: 2012 ACVIM Consensus Statement (Small Animal). *J Vet Intern Med.* 2013;27:1292–304.

24. Feldman EC. Effect of functional adrenocortical tumors on plasma cortisol and corticotropin concentrations in dogs. *J Am Vet Méd Assoc.* 1981;178:823–6.

25. Peterson ME, Gilbertson SR, Drucker WD. Plasma cortisol response to exogenous ACTH in 22 dogs with hyperadrenocorticism caused by adrenocortical neoplasia. *J Am Vet Med Assoc*. 1982;180:542–4.

26. Reusch CE, Feldman EC. Canine Hyperadrenocorticism Due to Adrenocortical Neoplasia: Pretreatment Evaluation of 41 Dogs. *J Vet Intern Med*. 1991;5:3–10.

27. Norman EJ, Thompson H, Mooney CT. Dynamic adrenal function testing in eight dogs with hyperadrenocorticism associated with adrenocortical neoplasia. *Vet Rec*. 1999;144:551–4.

28. Rijnberk A, Belshaw B. An alternative protocol for the medical management of canine pituitary-dependent hyperadrenocorticism. *Vet Rec.* 1988;122:486.

29. Liew CHV, Greco DS, Salman MD. Comparison of results of adrenocorticotropic hormone stimulation and low-dose dexamethasone suppression tests with necropsy findings in dogs: 81 cases (1985-1995). *J Am Vet Med Assoc.* 1997;211:322–5.

30. Vaughan MA, Feldman EC, Hoar BR, et al. Evaluation of twice-daily, low-dose trilostane treatment administered orally in dogs with naturally occurring hyperadrenocorticism. *J Am Vet Med Assoc.* 2008;232:1321–8.

31. Fracassi F, Corradini S, Floriano D, et al. Prognostic factors for survival in dogs with pituitary-dependent hypercortisolism treated with trilostane. *Vet Rec.* 2015;176:49.

32. Schofield I, Brodbelt DC, Wilson ARL, et al. Survival analysis of 219 dogs with hyperadrenocorticism attending primary care practice in England. *Vet Rec*. 2020;186:348–348.

33. Neiger R, Hurley KJ, Ramsey I, et al. Trilostane treatment of 78 dogs with pituitary-dependent hyperadrenocorticism. *Vet Rec*. 2002;150:799–804.

34. Arenas C, Melián C, Pérez-Alenza MD. Long-Term Survival of Dogs with Adrenal-Dependent Hyperadrenocorticism: A Comparison between Mitotane and Twice Daily Trilostane Treatment. *J Vet Intern Med*. 2014;28:473–80.

35. Alenza DP, Arenas C, Lopez ML, et al. Long-Term Efficacy of Trilostane Administered Twice Daily in Dogs With Pituitary-Dependent Hyperadrenocorticism. *J Am Anim Hosp Assoc.* 2006;42:269–76.

36. Hara Y. Transsphenoidal Surgery in Canines: Safety, Efficacy and Patient Selection. *Vet Medicine Res Reports.* 2020;11:1–14.

37. Massari F, Nicoli S, Romanelli G, et al. Adrenalectomy in dogs with adrenal gland tumors: 52 cases (2002–2008). *J Am Vet Med Assoc.* 2011;239:216–21.

38. Benchekroun G, Fornel-Thibaud PD, Piñeiro MIR, et al. Ultrasonography Criteria for Differentiating ACTH Dependency from ACTH *Independency* in 47 Dogs with Hyperadrenocorticism and Equivocal Adrenal Asymmetry. *J Vet Intern Med.* 2010;24:1077–85.

39. Rafael L. FVE Survey of the Veterinary Profession in Europe [Internet]. *Mirza & Nacey Research*; 2015 Apr. Available from: https://www.fve.org/cms/wp-content/uploads/FVE-Survey-ALL-280416_AMENDED-April-2016.pdf

Chapter 6

Comparison of methods to monitor dogs with hypercortisolism treated with trilostane

Stefania Golinelli, Viviani de Marco, Rodolfo Oliveira Leal,|
Andrea Barbarossa, Camilla Aniballi, Elisa Maietti, Antonio Maria Tardo,
Sara Galac, Federico Fracassi

J Vet Intern Med. 2021;1-12.

Dipartimento di Scienze Mediche Veterinarie,

Scuola di Agraria e Medicina Veterinaria,

Bologna

Abstract

Background: The use of adrenocorticotropic hormone stimulation test as method to monitor efficacy of trilostane treatment of hypercortisolism (HC) in dogs has been questioned.

Objectives: To evaluate and compare 12 methods with which to monitor efficacy of trilostane treatment in dogs with HC.

Animals: Forty-five client-owned dogs with HC treated with trilostane q12h.

Methods: Prospective cross-sectional observational study. The dogs were categorized as well-controlled, undercontrolled, and unwell through a clinical score obtained from an owner questionnaire. The ability to correctly identify trilostane-treatment control of dogs with HC with the following variables was evaluated: before trilostane serum cortisol (prepill), before-ACTH serum cortisol, post-ACTH serum cortisol, plasma endogenous ACTH concentrations, prepill/eACTH ratio, serum haptoglobin (Hp) concentration, serum alanine aminotransferase (ALT), gamma-glutamyl transferase (γGT) and alkaline phosphatase activity, urine specific gravity, and urinary cortisol : creatinine ratio.

Results: Ninety-four re-evaluations of 44 dogs were included; 5 re-evaluations of 5 unwell dogs were excluded. Haptoglobin was significantly associated with the clinical score ($P < .001$) and in the receiver operating characteristic analysis, Hp cutoff of 151 mg/dL correctly identified 90.0% of well-controlled dogs (specificity) and 65.6% of undercontrolled dogs (sensitivity). Alanine aminotransferase ($P = .01$) and γGT
($P = .009$) were significantly higher in undercontrolled dogs. Cutoff of ALT and γGT greater than or equal to 86 U/L and 5.8 U/L, respectively, were significantly associated with poor control of HC by trilostane.

Conclusions and Clinical Importance: Of all the 12 variables, Hp, and to a lesser degree ALT and γGT, could be considered additional tools to the clinical picture to identify well-controlled and undercontrolled trilostane-treated dogs.

Introduction

Naturally occurring Cushing's syndrome or hypercortisolism (HC) is a common endocrinopathy in dogs caused by chronic excessive glucocorticoid activity.[1] Trilostane has been the medical treatment of choice for pituitary and adrenal-dependent hypercortisolism (ADH) in the past 20 years.[2,3] The drug is a competitive inhibitor of the 3β-hydroxysteroid dehydrogenase/isomerase system required to synthesize cortisol, aldosterone, and androstenedione.[4] The appropriate dose and frequency of administration allow trilostane to control the clinical signs and the clinical-pathological abnormalities associated with HC.[5] For several years, the adrenocorticotropic hormone stimulation test (ACTHst) has been used to monitor trilostane treatment.5 However, over time, concerns have been raised regarding the reliability of this test.[6-8] The ACTHst has never been validated for trilostane monitoring purposes and the results strictly depend on the time of trilostane administration.[2-4,9-12] Recent evidence has supported a lack of correlation between post-ACTH administration serum cortisol concentration (post-ACTH) and clinical signs.[6,7] For these reasons, during the last decade, several methods to monitor trilostane treatment have been investigated.[7,8,13-17] Between all these possible monitoring tools, serum cortisol concentration before trilostane administration (prepill), urine specific gravity (USG), and haptoglobin (Hp), despite many limitations, showed the most promising results when investigated.[7,8,18] In particular, prepill showed a better correlation with the clinical picture in comparison with post-ACTH.7 However, when measuring 2 prepill taken an hour apart results significantly differ, thus questioning the ability of this method to replace the post-ACTH.[19] Finally, in 2020, different monitoring variables, such as USG, serial serum cortisol concentrations after trilostane administration (including prepill and post-ACTH), and the urine cortisol : creatinine ratio (UCCR), were evaluated taking the owner opinion on the course of clinical signs as the gold standard for clinical evaluation.[8] In the study, none of the previously cited variables was able to differentiate between well and undercontrolled dogs.[8] Haptoglobin concentration (Hp), a moderate acute phase protein, is higher in hypercortisolemic dogs.[20-22] Haptoglobin concentrations decline during trilostane treatment, suggesting a role of these variables as a monitoring tool to correctly identify trilostane treatment control.[13,15,1]

The conclusion of all the previously cited studies focused on the importance of the clinical evaluation to differentiate well-controlled from undercontrolled trilostane treated dogs. However, it is widely recognized that an assessment of an inexperienced owner or clinician can be unreliable at times. It is therefore mandatory to identify a laboratory monitoring method that can help to objectively discriminate between well-controlled and undercontrolled dogs treated with trilostane and it is able to identify overdosed dogs. The present study aimed to evaluate and compare the ability of 12 possible methods for monitoring trilostane treatment to correctly and objectively identify the clinical control in dogs classified as well-controlled, undercontrolled, and unwell. The clinical control was extrapolated from a previously standardized questionnaire completed by the dog owner along with the supervision of experienced veterinarians.[7]

Materials and Methods

Study design

A prospective cohort study involving client-owned dogs with a diagnosis of naturally occurring HC from 3 different veterinary hospitals (Veterinary Teaching Hospital of the University of Bologna, Veterinary Teaching Hospital of the University of Lisbon, Private Clinic Naya Especialidades of Sao Paulo de Brazil) from November 2017 to March 2020 was carried out.

Dogs

The diagnosis of HC was based on a combination of history (eg, polyuria and polydipsia, polyphagia and dermatological alterations), physical examination findings (eg, alopecia and abdominal enlargement), hematology (eg, lymphopenia, neutrophilia, and thrombocytosis), biochemistry (eg, abnormally high alanine aminotransferase [ALT], alkaline phosphatase [ALP], and gamma-glutamyl transferase [γGT]), urinalysis (eg, low USG and proteinuria), and endocrine testing (lowdose dexamethasone suppression test and ACTHst), were enrolled in the study.[23] A diagnosis of pituitary-dependent hypercortisolism (PDH) was made if any of the following criteria were met: a normal or high concentration of plasma endogenous adrenocorticotropic hormone concentration (eACTH; >5 pg/mL), cortisol concentration 8-hour post dexamethasone suppression above the lower limit of detection of the assay (1 mcg/dL or 28 nmol/L) and cortisol concentration 4-hour after dexamethasone suppression below the lower limit of detection of the assay (1 mcg/dL or 28 nmol/L) or less than 50% baseline, pituitary enlargement on magnetic resonance imaging (MRI) or computed tomography (CT; pituitary height-to-brain value > 0.31 x 10 2 mm 1),[24] or ultrasonographically bilaterally symmetric normal-sized or enlarged adrenal glands (width > 7.5 mm when not available breed-specific cutoff).[5] A diagnosis of ADH was made if the following criteria were met: low or undetectable eACTH (≤5 pg/
mL) and an ultrasonographically observed unilateral adrenal enlargement with atrophy of the contralateral adrenal gland. A diagnosis of concurrent PDH and the adrenal tumor was made if there was pituitary
enlargement on CT or MRI, not suppressed eACTH (>5 pg/mL), and the presence of an asymmetrically enlarged adrenal gland on CT or MRI with the contralateral gland within the normal limit.[25-30]
Dogs were included if they had been treated with trilostane twice daily (Vetoryl, Dechra, Shrewsbury, UK) at a stable dose for at least 3 weeks.
Dogs were excluded if they had any concurrent illness such as diabetes mellitus, acute or chronic kidney disease, azotemia, and symptomatic urinary tract infections (dogs with urological signs such as pollakiuria, hematuria, stranguria, and active urine sediment). Dogs were also excluded if treated with systemic or topical corticosteroids 1 month before the first evaluation or if they did not receive their trilostane dose the day before re-evaluation, if they showed neurological signs consistent with a suspicious large pituitary adenoma, or if they were anxious and aggressive. The sex, age, breed, body weight, number of previous re-evaluations, study center, and trilostane dosage at every re-evaluation were recorded. Dogs with more than 1 re-evaluation were included in the database more than once.

Clinical evaluation

A standardized questionnaire was used to assess the clinical picture of each dog, being completed by the owner with the help and supervision of the referring veterinarian.[7] The questionnaire consisted of 9 questions: 8 questions were used to assess thirst, urine volume, appetite, panting, exercise tolerance, coat quality, demeanor, gastrointestinal signs, and the overall owner impression regarding HC control, and 1 question was directed to identifying other signs of HC progression.[7] The questionnaire had a total score ranging from a minimum of 4 to a maximum of 28; a higher score implied greater severity of the HC clinical signs. No score was assigned to any answer, which was a possible sign of illness (eg, vomiting, diarrhea, anorexia). These answers were noted with the abbreviation PI (possible illness). Some answers (eg, answers regarding low activity) could have been classified with both the score and PI; when the classification was equivocal, they were noted with both categories (score and PI). Based on the total score, the dogs were classified as well-controlled (dogs

with good control of HC; scores from 4 to 11), undercontrolled (dogs with poor control of HC; score ≥ 12), or unwell (≥3 PI).[7]

Study protocol

Dogs were scheduled for consultation before receiving their morning trilostane dose. The owner of the dog was asked to bring the first urine sample of the day of the re-evaluation and the first urine sample of the day before. A first blood sample was taken immediately at the time of presentation, and each dog then received its dose of trilostane along with its usual meal provided by the owner. After 3 hours, an ACTHst was carried out by taking a blood sample (before ACTH administration cortisol [pre-ACTH]) and by administering IV 5 µg/kg of tetracosactide (Synacthen, Alfasigma S.P.A., Bologna, Italy) or Synacthen (Novartis, Buenos Aires, Argentina).[11] A third blood sample (post-ACTH) was taken 1 hour after synthetic ACTH administration.
Blood samples were collected from the jugular, cephalic, or saphenous veins. Sampling for the prepill, eACTH, Hp, ALT, ALP, and γGT was done at the time of presentation, and blood for the pre-ACTH and post-ACTH was taken 3 and 4 hours after the trilostane administration, respectively. Urine samples for the determination of USG and the UCCR were collected at home by the owner on the morning of the re-evaluation and the morning of the day before to avoid day-today variability. The owner was asked to keep the urine of the day before in the refrigerator until the re-evaluation day.

Dogs classified as unwell

Dogs identified as unwell based on the owner questionnaire score were excluded from further statistical analysis as they could not have a clinical score extrapolated from the questionnaire.

Analytical procedures

All the analytical procedures were carried out at the veterinary laboratory of the University of Bologna. The samples from Lisbon and Sao Paulo du Brazil were stored at -80°C and shipped overnight on dry ice to the veterinary laboratory of the University of Bologna. Blood samples for the determination of the eACTH were collected into EDTA-coated plastic tubes placed on ice. The samples were immediately centrifuged at 4°C, 500g for 8 minutes, and the plasma was immediately transferred to plastic tubes, stored at 4 C and analyzed within 8 hours or stored at -80°C and thawed immediately before analysis.[31] Blood samples for the determination of cortisol, Hp, ALT, ALP, and γGT were collected in serum separating tubes. Coagulated blood samples were centrifuged for 10 minutes at 3000g; the serum was immediately transferred to plastic tubes, stored at 4°C and analyzed the same day, or stored at -80°C and thawed immediately before analysis. Urine samples for the determination of USG and the UCCR were centrifuged for 10 minutes at 1000g. The USG was assessed using a previously calibrated refractometer immediately after the urine was centrifuged. The centrifuged urine was then transferred to plastic tubes, stored at 4°C and analyzed the same day, or stored at -80°C and thawed immediately before analysis.
Serum cortisol, urine cortisol (for UCCR determination), and eACTH concentrations were measured using a chemiluminescent enzyme immunoassay (Immulite 2000, Siemens Healthcare) which had been validated for dogs and is widely used in laboratories throughout the world.[32,33] Serum ALT, ALP, γGT activity, and serum Hp concentration and urine creatinine concentrations (for chemiluminescence UCCR determination) were measured using an automatic

analyzer (AU480, Beckman Coulter/Olympus, Brea, California). The Hp concentration was determined using an immunoturbidimetric method validated for dogs in the veterinary laboratory of the University of Bologna according to standard validation protocols, which included
intra-assay and interassay coefficients of variation <10% and linearity and recovery between 80% and 120%.[34] The reference range for a healthy dog of Hp concentration is 0 to 140 mg/dL.
Urine cortisol and creatinine concentrations were also measured individually using liquid chromatography-mass spectrometry (LC-MS)/MS. Cortisol was determined using 1.2 mL of urine to which cortisol-D4 internal
standard had previously been added, carrying out a cleanup step using a Waters Oasis SPE HLB cartridge according to a previously validated technique.35,36 For creatinine quantification, a 10 µL aliquot of urine sample was diluted 1:2000 in a 0.1% formic acid water : acetonitrile (50 : 50, vol/vol) solution containing the deuterated internal standard creatinine-D3.[37] The LC-MS/MS system consisted of aWaters Acquity UPLC binary pump, equipped with an Acquity BEH C18 (50 2.1 mm, 1.7 µm) column and coupled to a Waters Quattro Premier XE triple quadrupole mass spectrometer operating in (multiple reaction monitoring) MRM mode (Waters, Milford, Massachusetts). The specific transitions observed were: 363.1 > 120.8 for Cortisol and 367.1 > 120.7 for Cortisol-D4 (ESI); 114.1 > 44.1 for Creatinine and 117.1 > 47.0 for Creatinine-D3 (ESI+).

Ethical approval

The study was approved by the Scientific Ethical Committee of each participating University; each dog owner signed a written informed consent form before enrollment

Data analysis

The statistical unit was each dog's re-evaluation, as during the study period a dog could be evaluated more than once. Shapiro-Wilk test was used to assess the normality of all the continuous variables. Non-normally distributed variables were reported as median and interquartile range (IQR), while normally distributed variables were reported as mean ± SD. The differences between USG and UCCR measures taken the day before and the day of re-evaluation were assessed by either paired t test when they were normally distributed or by Wilcoxon signed-rank test when they were non-normally distributed. In the case of nonsignificant differences, only re-evaluation USG and UCCR (greater number of the sample) were included in the subsequent analysis. Univariate linear regression analysis was used to assess the association between the total score and the other variables. Since a dog could be included more than once, robust SEs allowing for intragroup correlation were calculated with vce(cluster) Stata command. Results were reported as regression coefficient (b) and 95% confidence interval (95% CI). Multiple regression analyses were used
to adjust the association between the total score and each monitoring method for the possible confounding factors (study center, number of previous re-evaluations, and trilostane dosage). Univariate and multiple logistic regression analyses were used to assess the association between poor control (dependent variable) and the monitoring methods (independent variables). Robust SEs allowing for intragroup correlation were calculated and results were reported as odds ratio (OR) and 95% CI. Variables with $P < .1$ in multiple logistic regression analysis were further investigated using receiver operating characteristic (ROC) curves analysis to evaluate their discriminative ability. For the variables with an area under the ROC curve (AUC), ≥0.75 optimal cutoffs were determined to maximize the specificity while maintaining the sensitivity ≥50%, therefore

reducing the likelihood of false-positive results. A multiple forward stepwise regression analysis was performed to investigate if 2 or more monitoring variables were able to predict the clinical score. R2 coefficient of determination was calculated to assess the model's goodness of fit, that is, variables' predictive ability. A sensitivity analysis including only the first re-evaluation was performed to assess the robustness of the results. Univariate and multiple linear regressions were used to assess the association between the total score and each monitoring method. The Mann-Whitney U test was carried out to compare monitoring methods between well-controlled and undercontrolled dogs. Variables with $P < .1$ were further investigated using ROC curves analysis to evaluate their discriminative ability and to determine optimal cutoffs to maximize the specificity while maintaining the sensitivity ≥50%. Statistical analyses were carried out using commercially available Stata statistical software version 15 (Stata Statistical Software: Release 15. College Station, Texas: StataCorp LLC. StataCorp. 2017). A P value of <.05 was considered significant.

Results

Ninety-nine re-evaluations of 45 dogs were included in the study. Fifty-three re-evaluations were performed at the Veterinary Teaching Hospital of the University of Bologna, 23 at the Private Clinic Naya Especialidades of Sao Paulo de Brazil, and 23 at the Veterinary Teaching Hospital of the University of Lisbon.

Dogs

There were 20 male dogs (14 intact and 6 neutered) and 25 female dogs (13 intact and 12 neutered). At the first presentation, the median age was 11 years (IQR, 9.5-14), and median body weight was 10.5 kg (IQR, 7.1-15.1). Nineteen mixed breed dogs, 5 Maltese, 4 Poodles, 3 each Dachshund, Shih-Tzu, Yorkshire Terrier, and 1 each of Riesenshnauzer, Pinscher, Boston Terrier, French Bulldog, Boxer, Beagle, Lhasa Apso, and Newfoundland were included in the study. One dog was diagnosed with ADH, 1 dog with PDH and an adrenal tumor, and 43 dogs with PDH. The median dose of trilostane at the time of
all 99 tests was 1 mg/kg q12h (IQR, 0.66-1.31). The median time between diagnosis and the first re-evaluation was 17 weeks (IQR, 5.9-83). The minimum time between consecutive re-evaluations was 3.6 weeks. Twenty-six dogs had more than 1 consecutive re-evaluation: 12 had 2 re-evaluations, 6 had 3 re-evaluations, 5 had 4 reevaluations, and 3 had 6 re-evaluations. The time between each reevaluation (weeks) is reported in Table 1.

TABLE 1 Median and interquartile of time between each re-evaluation (weeks)

Interval between re-evaluations	Median(weeks)	IQR (weeks)
1°-2°	9.2	4.9-17
2°-3°	5.9	4.4-12
3°-4°	5.6	3.9-6.5
4°-5°	6.7	4.3-13
5°-6°	8	6.1-9.9

Abbreviation: IQR, interquartile range.

The prepill, pre-ACTH, post-ACTH, eACTH, prepill/eACTH ratio (prepill/eACTH), Hp, ALT, γGT, ALP, UCCR of the re-evaluation day obtained using chemiluminescence (day-of-re-evaluation CUCCR) and USG of the re-evaluation day

(day-of-re-evaluation USG) were measured for all 99 re-evaluations, except in 1 dog in which the urine samples were not available. The UCCR measured with chemiluminescence of the day before the re-evaluation (day-before CUCCR) and the USG of the day before the re-evaluation (day-before USG) were measured in 78 re-evaluations. The LC-MS/MS UCCR of the re-evaluation day (day-of-reevaluation LUCCR) was measured in 76 tests. LC-MS/MS UCCR of the day before (day-before LUCCR) was measured in 63 tests.

Clinical avaluation

Based on the owner questionnaire, 31 dogs' re-evaluations were classified as well-controlled, 63 as undercontrolled, and 5 as unwell. The 5 unwell dogs were excluded from further statistical analysis. The mean score for the 94 owner questionnaires was 13.6 ± 3.7, with a minimum of 6 and a maximum of 22. The mean score was 13.1 ± 4.1 at the University of Bologna,13.1 ± 2.7 at the Private Clinic Naya Especialidades of Sao Paulo de Brazil, and 15.3 ± 3.6 at the University of Lisbon, differences not statistically significant (P = .14).

Association between monitoring methods and total score

Simple and adjusted associations between monitoring methods and owner's score are reported in Table 2.

TABLE 2 Association between monitoring method variables and clinical score: results from simple and multiple linear regression analysis

	Simple associations			Adjusted associations		
Variables	***b***	**95% CI**	***P* value**	***b***	**95% CI**	***P* value**
ALT	0.008	0.004-0.013	**.001**	0.007	0.004-0.011	**<.001**
γGT	0.018	0.001-0.036	**.04**	0.024	0.010-0.038	**.002**
ALP	0.002	0.001-0.003	**.003**	0.002	0.001-0.003	**.001**
Hp	0.029	0.015-0.044	**<.001**	0.029	0.016-0.041	**<.001**
Day-of-re-evaluation USG	−0.097	−0.152 to −0.041	**.001**	−0.084	−0.139 to −0.031	**.003**
Day-of-re-evaluation CUCCR	0.004	−0.001 to 0.008	.06	0.005	0.001-0.009	**.04**
Day-of-re-evaluation LUCCR[a]	0.015	0.003-0.027	**.02**	0.016	0.001-0.032	**.05**
Prepill cortisol	0.450	0.168-0.725	**.002**	0.517	0.238-0.795	**.001**
Pre-ACTH cortisol	0.430	−0.016 to 0.875	.06	0.463	0.080-0.846	**.02**
Post-ACTH cortisol	0.310	0.011-0.513	**.004**	0.301	0.128-0.473	**.001**
eACTH	−0.001	−0.004 to 0.002	.59	−0.002	−0.006 to 0.002	.26
Prepill/eACTH (0.01 unit increase)	0.071	−0.047 to 0.19	.23	−0.083	−0.023 to 0.189	.12

Note: The regression coefficient, 95% confidence interval, and *P* value of each variable are reported. Adjustment factors included study center, number of previous re-evaluations, and trilostane dosage; in bold *P* value < .05.
Abbreviations: 95% CI, 95% confidence interval; γGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; *b*, regression coefficient; day-of-re-CUCCR, chemiluminescence urine cortisol : creatinine ratio of the re-evaluation day; day-of-re-evaluation USG, urine specific gravity of the re-evaluation day; day-of-re-LUCCR, liquid chromatography-tandem mass spectrometry urine cortisol : creatinine ratio of the re-evaluation day; eACTH, endogenous ACTH concentration; Hp, haptoglobin concentration; post-ACTH, post-ACTH administration cortisol concentration; pre-ACTH cortisol, before ACTH administration cortisol concentration; prepill cortisol, before trilostane administration serum cortisol concentration; prepill/eACTH, prepill/eACTH ratio.
[a]Available for 76/94 observations.

In multiple regression analyses adjusted for trilostane dosage, number of previous reevaluations, and study center, all the variables except the eACTH and prepill/eACTH ratio were significantly associated with the total score. The score decreased with the increase in USG (b = -0.08, 95% CI = -0.14 to -0.03, P = .003), while it increased with the increase in the other variables.

Association between monitoring methods and inadequate control

Simple and adjusted associations between monitoring methods and inadequate control are reported in Table 3.

TABLE 3 Association between monitoring method variables and inadequate control: results from univariate logistic regression analysis

	Simple associations			Adjusted associations		
Variables	OR	95% CI	*P* value	OR	95% CI	*P* value
ALT	1.011	1.000-1.022	**.05**	1.008	0.999-1.017	.07
γGT	1.012	0.995-1.030	.17	1.010	0.999-1.021	.07
ALP	1.001	1.000-1.002	.09	1.000	1.000-1.001	.2
Hp	1.013	1.005-1.022	**.002**	1.010	1.002-1.019	**.01**
Day-of-re-evaluation USG	0.958	0.928-0.989	**.009**	0.964	0.936-0.993	**.02**
Day-of-re-evaluation CUCCR	1.006	1.000-1.011	**.04**	1.006	1.001-1.011	**.01**
Day-of-re-evaluation LUCCR[a]	1.069	0.996-1.147	.06	1.109	0.982-1.252	.1
Prepill cortisol	1.279	1.008-1.622	**.04**	1.327	1.051-1.675	**.02**
Pre-ACTH cortisol	1.211	0.974-1.505	.08	1.201	0.954-1.513	.12
Post-ACTH cortisol	1.132	0.993-1.291	.06	1.116	0.993-1.254	.06
eACTH	1.000	0.998-1.002	.74	1.000	0.997-1.003	.88
Prepill/eACTH (0.01 unit increase)	1.006	0.947-1.069	.84	1.021	0.966-1.078	.46

Note: The odd ratio, 95% confidence interval, and *P* value of each variable are reported. Adjustment factors included study center, number of previous re-evaluations, and trilostane dosage; in bold *P* value < .05.
Abbreviations: 95% CI, 95% confidence interval; γGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; day-of-re-evaluation CUCCR; chemiluminescence urine cortisol : creatinine ratio of the re-evaluation day; day-of-re-evaluation LUCCR, liquid chromatography-tandem mass spectrometry urine cortisol : creatinine ratio of the re-evaluation day; day-of-re-evaluation USG, urine specific gravity of the re-evaluation day; eACTH, endogenous ACTH concentration; Hp, haptoglobin concentration; OR, odds ratio; post-ACTH, post-ACTH administration cortisol concentration; pre-ACTH cortisol, before ACTH administration cortisol concentration; prepill cortisol, before trilostane administration serum cortisol concentration; prepill/eACTH, prepill/eACTH ratio.
[a]Available for 76/94 observations.

In multiple regression analysis adjusted for confounding factors serum Hp (OR = 1.01, 95% CI = 1.00-1.02, P = .01), day-of-re-evaluation USG (OR = 0.96, 95% CI = 0.94-0.99, P = .02), day-of-re-evaluation CUCCR (OR = 1.01, 95% CI = 1.00-1.01, P = .01), and prepill (OR = 1.33, 95% CI = 1.05-1.68, P = .02) were significantly associated to poor control. Alanine aminotransferase, γGT, ALP, dayof- re-evaluation LUCCR, pre-ACTH, and post-ACTH did not reach statistical significance. The AUC of the variables is reported in
Table 4.

TABLE 4 ROC curve analysis results

Variables	AUC	95% CI	Cutoff	Specificity %	Sensitivity %	Accuracy %
ALT (U/L)	**0.76**	0.66-0.86	120	90	56.3	67
γGT (U/L)	0.71	0.60-0.83				
Hp (mg/dL)	**0.75**	0.65-0.85	151	90	65.6	73.4
Day-of-re-evaluation USG	0.65	0.53-0.77				
Day-of-re-evaluation CUCCR	0.65	0.53-0.77				
Day-of-re-evaluation LUCCR[a]	0.66	0.52-0.80				
Prepill cortisol (μg/dL)	0.65	0.53-0.77				
Post-ACTH cortisol (μg/dL)	0.59	0.47-0.71				

Note: The AUC and 95% CI of all variables are reported. The cutoff value, specificity, sensitivity, and accuracy of each variable with an AUC ≥ 0.75 are reported; in bold, AUC ≥ 0.75.
Abbreviations: 95% CI, 95% confidence interval; γGT, gamma-glutamyl transferase; ALT, alanine aminotransferase; AUC, area under the ROC curve; day-of-re-evaluation CUCCR; chemiluminescence urine cortisol : creatinine ratio of the re-evaluation day; day-of-re-evaluation LUCCR, liquid chromatography-tandem mass spectrometry urine cortisol : creatinine ratio of the re-evaluation day; day-of-re-evaluation USG, urine specific gravity of the re-evaluation day; Hp, haptoglobin concentration; post-ACTH, post-ACTH administration cortisol concentration; prepill cortisol, before trilostane administration serum cortisol concentration.
[a]Available for 76/94 observations.

In ROC analysis, only ALT and Hp showed a good discriminative ability (AUC ≥ 0.75). Hp ≥151 mg/dL correctly identified 90.0% of well-controlled dogs (specificity) and 65.6% of undercontrolled
dogs (sensitivity) with an overall accuracy of 73.4% (69/94) while ALT ≥ 120 U/L showed a specificity of 90% and a sensitivity of 56.3%, with an overall accuracy of 67% (63/94).

Clinical score predicting model

In the multiple regression analysis, Hp was the best predictor of the clinical score (R2 = 0.359). Using forward stepwise regression analysis, the addition of prepill to the multiple model resulted in significance (P = .04) but the increase in goodness of fit was little (R2 = 0.382). No other variables had a significant added value.

Sensitivity analysis

Forty-three dogs were included in the sensitivity analysis at the first re-evaluation after diagnosis. Simple and adjusted associations between each monitoring method and the total score are reported in Table 5.

TABLE 5 Association between monitoring method variables and clinical score: results from simple and multiple linear regression analysis of data of the first re-evaluation

	Simple associations			Adjusted associations		
Variables	*b*	95% CI	*P* value	*b*	95% CI	*P* value
ALT	0.004	−0.002 to 0.011	.2	0.005	−0.002 to 0.011	.17
γGT	0.023	−0.002 to 0.048	.07	0.028	0.002-0.053	**.03**
ALP	0.001	−0.001 to 0.003	.14	0.002	−0.001 to 0.003	.07
Hp	0.039	0.022-0.056	**<.001**	0.038	0.021-0.056	**<.001**
Day-of-re-evaluation USG	−0.045	−0.157 to −0.057	**.38**	−0.057	−0.158 to −0.043	.25
Day-of-re-evaluation CUCCR	0.010	0.002-0.019	**.02**	0.013	0.005-0.022	**.003**
Day-of-re-evaluation LUCCR[a]	0.015	−0.010 to 0.040	.23	0.018	0.008-0.044	.16
Prepill cortisol	0.554	0.186-0.923	**.004**	0.647	0.267-1.160	**.001**
Pre-ACTH cortisol	0.632	0.185-1.079	**.007**	0.717	0.106-0.682	**.002**
Post-ACTH cortisol	0.323	0.026-0.620	**.03**	0.394	0.106-0.682	**.009**
eACTH	−0.001	−0.006 to 0.004	.71	−0.002	−0.007 to 0.003	.45
Prepill/eACTH (0.01 unit increase)	0.015	−0.117 to 0.146	.82	−0.032	−0.100 to 0.165	.63

Note: The regression coefficient, 95% confidence interval, and *P* value of each variable are reported. Adjustment factors included study center and trilostane dosage; in bold *P* value < .05.
Abbreviations: 95% CI, 95% confidence interval; γGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; *b*, regression coefficient; day-of-re-CUCCR, chemiluminescence urine cortisol : creatinine ratio of the re-evaluation day; day-of-re-evaluation USG, urine specific gravity of the re-evaluation day; day-of-re-LUCCR, liquid chromatography-tandem mass spectrometry urine cortisol : creatinine ratio of the re-evaluation day; eACTH, endogenous ACTH concentration; Hp, haptoglobin concentration; post-ACTH, post-ACTH administration cortisol concentration; pre-ACTH cortisol, beforeACTH administration cortisol concentration; prepill cortisol, before trilostane administration serum cortisol concentration; prepill/eACTH, prepill/eACTH ratio.
[a]Available for 37/43 observations.

In multiple regression analysis adjusted for trilostane dosage and study center, Hp, γGT, day-of-re-evaluation CUCCR, prepill, pre-ACTH, and post-ACTH were still significantly associated with the total
score. On the contrary, ALT, ALP, day-of-re-evaluation USG, and LUCCR did not reach statistical significance.
Among the 43 dogs included, 31 (72.1%) were undercontrolled. The monitoring methods were compared between wellcontrolled and undercontrolled groups by the Mann-Whitney U test (Table 6).

TABLE 6 Median and interquartile range of monitoring parameters according to well-controlled and undercontrolled groups of data of the first re-evaluation

Variables	Well-controlled (n = 12)	Undercontrolled (n = 31)	*P* value
ALT (U/L)	71 (53-85)	118 (83-210)	**.01**
γGT (U/L)	3 (1.6-5)	6.6 (3.9-13)	**.009**
ALP (U/L)	367 (90-731)	298 (112-774)	.61
Hp (mg/dL)	119 (85-141)	173 (142-187)	**.005**
Day-of-re-evaluation USG	1023 (1013-1035)	1026 (1014-1038)	.82
Day-of-re-evaluation CUCCR	84 (42-129)	90 (112-774)	.24
Day-of-re-evaluation LUCCR[a]	4.1 (2.6-9.1)	4 (2.9-6.9)	.67
Prepill cortisol (μg/dL) (nmol/L)	2.6 (2-4.2) 71.7 (55.2-115.9)	3.6 (2.4-5.5) 99.3 (66.2-151.7)	.24
Pre-ACTH cortisol (μg/dL) (nmol/L)	2.7 (2-3.5) 74.5 (55.2-96.6)	2.5 (1.9-4.9) 69 (52.4-135.2)	.74
Post-ACTH cortisol (μg/dL) (nmol/L)	4.6 (3.7-6.2) 126.9 (102.1-71.1)	6.2 (3.3-8.8) 171.1 (91-242.8)	.37
eACTH (pg/mL)	80 (21-188)	90 (37-125)	.79
Prepill/eACTH	0.05 (0.01-0.13)	0.05 (0.03-0.11)	.77

Note: Comparison between groups with Mann-Whitney *U* test at the first re-evaluation.
Abbreviations: γGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; day-of-re-CUCCR, chemiluminescence urine cortisol : creatinine ratio of the re-evaluation day; day-of-re-evaluation USG, urine specific gravity of the re-evaluation day; day-of-re-LUCCR, liquid chromatography-tandem mass spectrometry urine cortisol : creatinine ratio of the re-evaluation day; eACTH, endogenous ACTH concentration; Hp, haptoglobin concentration; post-ACTH, post-ACTH administration cortisol concentration; pre-ACTH cortisol, before ACTH administration cortisol concentration; prepill cortisol, before trilostane administration serum cortisol concentration; prepill/eACTH, prepill/eACTH ratio.
[a]Available for 37/43 observations.

Alanine aminotransferase, γGT, and Hp were significantly higher in undercontrolled dogs (Figures 1-3).

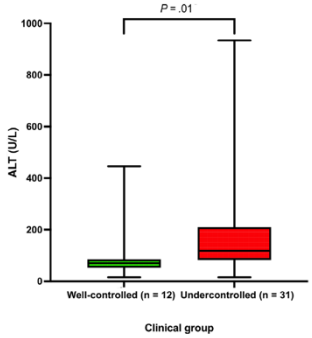

FIGURE 1 Box and whisker plots of ALT data of the first re-evaluation divided into 2 groups of clinical control: well-controlled HC dogs (n = 12) and undercontrolled HC dogs (n = 31). The lower and upper boundaries of the box represent the first and third quartiles of the data, respectively, with the line within the box representing the median. The whiskers represent the 5th to 95th percentile. Significantly different results are indicated by connecting horizontal lines with the *P* values shown above. ALT, alanine aminotransferase; HC, hypercortisolism

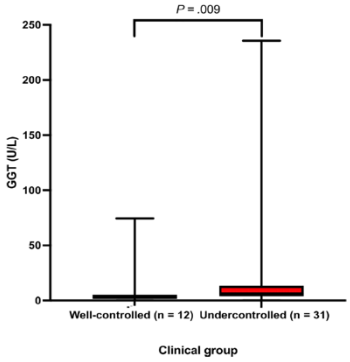

FIGURE 2 Box and whisker plots of γGT data of the first re-evaluation divided into 2 groups of clinical control: well-controlled HC dogs (n = 12) and undercontrolled HC dogs (n = 31). The lower and upper boundaries of the box represent the first and third quartiles of the data, respectively, with the line within the box representing the median. The whiskers represent the 5th to 95th percentile. Significantly different results are indicated by connecting horizontal lines with the *P* values shown above. γGT, gamma-glutamyl transferase; HC, hypercortisolism

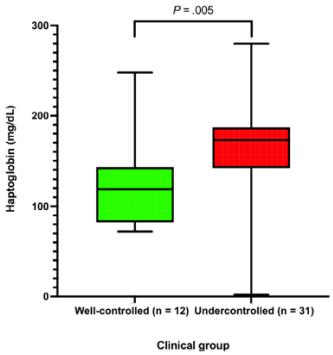

FIGURE 3 Box and whisker plots of Haptoglobin (Hp) data of the first re-evaluation divided into 2 groups of clinical control: well-controlled HC dogs (n = 12) and HC dogs undercontrolled (n = 31). The lower and upper boundaries of the box represent the first and third quartiles of the data, respectively, with the line within the box representing the median. The whiskers represent the 5th to 95th percentile. Significantly different results are indicated by connecting horizontal lines with the *P* values shown above. HC, hypercortisolism

All the 3 variables showed a good discriminative ability, and optimal cutoffs were ≥86 U/L, ≥5.8 U/L, and ≥151 mg/dL for ALT, γGT, and Hp, respectively (Table 7).

TABLE 7 ROC curve analysis results of data of the first re-evaluation

Variables	AUC	95% CI	Cutoff	Specificity %	Sensitivity %	Accuracy %
ALT (U/L)	0.76	0.59-0.93	86	83.3	71	74.4
γGT (U/L)	0.76	0.59-0.93	5.8	83.3	67.7	72.1
Hp (mg/dL)	0.78	0.61-0.94	151	91.7	64.5	72.1

Note: The AUC and 95% CI of all variables are reported. The cutoff value, specificity, sensitivity, and accuracy of each variable with an AUC ≥ 0.75 are reported.
Abbreviations: 95% CI, 95% confidence interval; γGT, gamma-glutamyl transferase; ALT, alanine aminotransferase; AUC, area under the ROC curve; Hp, haptoglobin concentration.

Unwell dogs

Five dogs were classified as unwell based on the owner questionnaire score. Just in 1/5 dogs, iatrogenic hypoadrenocorticism was diagnosed based on clinical signs (anorexia, vomit, and lethargy), the result of biochemistry (increase in serum potassium, decrease in serum sodium), endocrine evaluations (ACTHst and eACTH), and abdominal ultrasound.This dog had prepill and post-ACTH lower than 1.4 µg/dL while the
eACTH was >1250 pg/mL and the abdominal ultrasound showed a hypoechoic enlarged adrenal gland with a hyperechoic surrounding fatty tissue. In the middle of the fatty tissue, mild abdominal effusion was
present. Based on ultrasound findings and the acute development of clinical signs, adrenal necrosis was suspected. The dog needed glucocorticoid and mineralocorticoid replacement treatment.
All the other dogs classified as unwell had prepill and post-ACTH over the limit of 1.4 µg/dL.
None of these 4 dogs developed trilostane overdose in the follow-ups.

Low prepill and post-ACTH cortisol

There were 10 dogs with prepill less than 1.4 µg/dL. Three of these 8 dogs were classified as undercontrolled, the remaining ones as wellcontrolled from the owner questionnaire. One of these dogs had also the post-ACTH less than 1.4 µg/dL and was classified as undercontrolled. All of these dogs had Hp concentration < 151 mg/dL, 6/8 dogs had γGT < 5.8 U/L, and 5/8 dogs had <86 U/L.
One dog had the post-ACTH less than 1.4 µg/dL with prepill >1.4 µg/dL, γGT < 5.8 U/L, and increased ALT > 86 U/L. The dog was classified as undercontrolled based on the questionnaire score. None of these 9 dogs developed trilostane overdose in the follow-ups when available. All these results are reported in Table 8.

TABLE 8 Low prepill and post-ACTH cortisol results (<1.4 mg/dL)

Dog	Prepill cortisol (µg/dL)	Post-ACTH cortisol (µg/dL)	ALT (U/L)	γGT (U/L)	Hp (mg/dL)	Score	Clinical control
1	1.13	1.2	36	3.6	86	15	Undercontrolled
2	1.1	6.2	96	3.9	125	13	Undercontrolled
3	1.32	5.54	53	1.3	80	9	Well-controlled
4	1.32	3.44	85	5.4	137	10	Well-controlled
5	2.23	1.28	282	6.1	144	14	Undercontrolled
6	<1	3.31	38	2.6	118	11	Well-controlled
7	<1	2.38	105	156.9	89	15	Undercontrolled
8	<1	5.73	31	4.1	107	7	Well-controlled
9	<1	1.69	97	1.2	134	9	Well-controlled

Abbreviations: γGT, gamma-glutamyl transferase; ALT, alanine aminotransferase; Hp, haptoglobin concentration; post-ACTH, post-ACTH administration cortisol concentration; prepill cortisol, before trilostane administration serum cortisol concentration.

Discussion

The present study aimed to identify a laboratory variable able to objectively identify clinical well-controlled from undercontrolled HC dogs treated with trilostane. Indeed, unreliable owner observations, inexperienced clinician assessment, moderation of dose adjustments, and potentially early warning of an overdose make it mandatory to find an objective monitoring tool for trilostane-treated dogs with HC. This research investigated 12 possible monitoring methods in a population of dogs with HC treated with trilostane, whose clinical control was defined based on a score obtained from an owner questionnaire.[7]

Hp concentration, a moderate acute phase protein, increases in hypercortisolemic state and decreases during trilostane treatment.[14,16,20-22] However, when compared to the ACTHst, Hp did not show any additional information in assessing the clinical control.[14,16]

Our investigation results revealed that increased serum Hp concentrations were significantly associated with poor control of HC. This significance of Hp was maintained, also when only the data of the first re-evaluation was included in the statistical analysis and the association between the monitoring method and clinical score was evaluated including the possible influence of the different study centers, the number of previous re-evaluations, and the trilostane dosage. Therefore, our findings suggest that Hp was the best predictor among the 12 monitoring methods. However, the overlap between Hp concentration in well-controlled and undercontrolled dogs makes mandatory further studies about this monitoring tool.

A recent study showed similar results and hypothesized minor influence of short-term cortisol changes on Hp concentration.[18] The idea of serum haptoglobin as a reflection of the cortisol concentration of the last time period ("cortisol history"), as serum fructosamine reflects the glucose concentrations in the previous 7 to 14 days ("glucose history"), still needs to be demonstrated.[38] The research on the effect of exogenous corticosteroids on Hp concentration has shown that plasma Hp concentration started to increase the day after the first glucocorticoid administration and was still above the baseline value 14 days after[20,21] No information is available regarding the duration of endogenous cortisol Hp induction in dogs; however, it

seemed to be lesser than that of exogenous glucocorticoids.[22] Additional studies on monitoring Hp trends starting from HC diagnosis, and following trilostane treatment, are needed. Because Hp is a positive acute-phase protein, results could be biased if a dog has a concomitant inflammatory state (ie, urinary tract infection). For this reason, it is advisable to interpret Hp concentrations individually, taking into account the clinical picture of the dog monitored.

Next to Hp, prepill cortisol, γGT, day-of-re-evaluation USG, and CUCCR were all significantly associated with the clinical score also when only data of the first re-evaluation and the influence of study center, and trilostane dosage were considered. However, when the ability of the variables to discriminate well and undercontrolled dogs was assessed just on the data of the first re-evaluation and with no influence of repeated measures, besides Hp, only γGT and ALT gave consistent results (AUC $\geq$ 0.75).

Alanine aminotransferase and γGT are increased in HC dogs.[5] Results of our study showed that undercontrolled HC dogs had significantly higher ALT and γGT in comparison to well-controlled ones. In particular, values of ALT and γGT equal or greater than 86 U/L and 5.8 U/L, respectively, were significantly associated with a poor trilostane treatment control. The biological relevance of these data is unknown so far and the large overlap between the concentrations of these 2 variables in well-controlled and undercontrolled dogs makes these results to be taken with caution. Day-of-re-evaluation CUCCR and LUCCR and USG, eACTH, prepill/eACTH, pre-ACTH, and post-ACTH were not able to correctly identify the correct clinical control in trilostane treated dogs, as previously reported.[7,8,14,16,17] The inability to evaluate trilostane monitoring with the UCCR was ascribed to the analytical method which detects cortisol and its metabolites.[15] We measured UCCR with

LC-MS/MS to avoid the possible interference with urinary cortisol metabolites and precursors, however, despite that, both the chemiluminescence and LC-MS/MS UCCR were not able to differentiate the 2 categories of clinical control, pointing out that the results are independent of the analytical method used.[39]

The concentration of eACTH increases during trilostane treatment due to the loss of negative feedback regarding the cortisol concentration to the pituitary.[40,41] It was hypothesized that prepill/ eACTH and eACTH could reflect the cortisol concentration during trilostane treatment and could be used as methods to monitor it. However, according to our results,

the eACTH, and the prepill/ eACTH ratio, failed to differentiate between the well-controlled and undercontrolled dogs, which is in agreement with previous reports.[17]

The ACTHst was not able to correctly identify the undercontrolled dogs according to previous investigations.[7,8] When only data of the first re-evaluation were analyzed to assess the robustness of the results, prepill and day-to-re-evaluation USG failed to significantly discriminate well and undercontrolled dogs. The limitation of these 2 possible monitoring tools has already been described in the literature, confirming the low reliability of these variables to correctly evaluate the trilostane treatment control of HC dogs.[6,8,18]

Our research has some limitations. First, the time of the day of prepill sampling was not standardized, and this, as seen in other studies, could have potentially influenced the results.[7,8] However, all reevaluations were carried out in a routine clinical setting in which the exact time of the sampling is not typically standardized. The second limitation concerned the subjective nature of the questionnaire used for the clinical evaluation. A standardized questionnaire, which had already been used in previous studies, was chosen.[7,18] However, the questionnaire was based on owner observations, and over or underestimation of trilostane treatment efficacy could not be excluded. Still, even when a questionnaire is not used, the evaluation of a dog on trilostane treatment is based partially on the owner's opinion about some signs (ie, polyuria and polydipsia, polyphagia, etc). The survey aimed to evaluate this information in the most objective way possible but still, the owner observation remains a subjective way to interpret the dog clinical control. In our investigation, the frequency of reevaluations was determined by the attending clinicians and was not standardized. There might be an inherent bias toward the less stable dogs (ie, well-controlled dogs got fewer re-evaluations). Another possible limitation is the inclusion of just 1 dog with ADH in the study in comparison with other studies. This can be justified by the fact that the majority of ADH dogs are not treated with trilostane because adrenal surgery is always the first choice of treatment suggested to the owners in the author's working hospital. Last, blood samples for the determination of cortisol, Hp, ALT, ALP, and γGT were collected in serum separating tubes. While some studies have shown no influence of separating gel on cortisol, ALT, ALP, and γGT, no evidence is available about Hp and the authors cannot rule out an impact on Hp results.[42,43]

Finally, conclusions regarding the reliability of the methods analyzed to recognize overdosed dogs could not be drawn from the present data. This research identified just 1 dog with an excess of trilostane. This result could have been the consequence of the presence of fewer overdosed dogs in general, probably because the initial recommended trilostane dose today is much lower as compared to the past.[44] Moreover, many dogs in this investigation had been treated for only a short period of time and were strictly monitored for study purposes; this could have influenced the possibility of showing an excess of trilostane and may not have reflected the number of overdose dogs which can be seen in the first re-evaluation. None of the dogs with prepill or post-ACTH less than 1.4 μg/dL developed a trilostane overdose when follow-up was available. Previously published research showed a failure to respond adequately to ACTH stimulation at a particular time point (when trilostane is at its peak) does not always reflect a trilostane overdose.[12] At the same time, data are lacking about the ability of prepill to identify overdosed dogs.

Conclusions about the performance of these tools to recognize overcontrolled dogs are not possible.

In conclusion, this was the first study comparing 12 methods to monitor trilostane treatment in dogs with HC. Specifically, an integrated and fully comprehensive evaluation of the known monitoring methods available to date was carried out.

Based on the present results, good history taking (and physical examination) cannot be replaced by a laboratory variable at this moment. Hp, and to a lesser degree ALT and γGT, could be considered additional tools to the clinical picture to correctly identify well-controlled and undercontrolled trilostane-treated dogs. However, none of these variables is able to identify the overcontrolled dogs

References

1. Schofield I, Brodbelt DC, Niessen SJ, et al. Development and internal validation of a prediction tool to aid the diagnosis of Cushing's syndrome in dogs attending primary-care practice. *J Vet Intern Med.* 2020;34:2306-2318.

2. Ruckstuhl NS, Nett CS, Reusch CE. Results of clinical examinations, laboratory tests, and ultrasonography in dogs with pituitarydependent hyperadrenocorticism treated with trilostane. *Am J Vet Res*. 2002;63:506-512.

3. Neiger R, Ramsey I, O'Conner J, et al. Trilostane treatment of 78 dogs with pituitary-dependent hyperadrenocorticism. *Vet Rec*. 2002;150: 799-804.

4. Potts GO, Creange JE, Hardong HR, et al. Trilostane, an orally active inhibitor of steroid biosynthesis. *Steroids.* 1978;32:257-267.

5. Behrend EN. Canine hyperadrenocorticism. In: Feldman EC, Nelson RW, Reusch CE, et al., eds. Canine and Feline Endocrinology. 4th ed. St. Louis, MO: Elsevier Saunders; 2015:377-451.

6. Boretti FA, Holzthuem J, Reusch CE, et al. Lack of association between clinical sings and laboratory parameters in dogs with hyperadrenocorticism before and during trilostane treatment. *Schweiz Arch Tierheilkd.* 2016;158:631-638.

7. Macfarlane L, Parkin T, Ramsey I. Pre-trilostane and three-hour post trilostane cortisol to monitor trilostane therapy in dogs. *Vet Rec*. 2016;179:597-601.

8. Arenas Bermejo C, Pérez Alenza D, García San José P, et al. Laboratory assessment of trilostane treatment in dogs with pituitarydependent hyperadrenocorticism. *J Vet Intern Med*. 2020;34:1413-1422.

9. Bell R, Neiger R, McGrotty Y, Ramsey IK. Study of the effects of once daily doses of trilostane on cortisol concentrations and responsiveness to adrenocorticotrophic hormone in hyperadrenocorticoid dogs. *Vet Rec.* 2006;159:277-281.

10. Bonadio CM, Feldman EC, Cohen TA, Kass PH. Comparison of adrenocorticotropic hormone stimulation test results started 2 versus 4 hours after trilostane administration in dogs with naturally occurring hyperadrenocorticism. *J Vet Intern Med.* 2014;28:239-1243.

11. Griebsch C, Lehnert C, Williams GJ, Failing K, Neiger R. Effect of trilostane on hormone and serum electrolyte concentrations in dogs with pituitary-dependent hyperadrenocorticism. *J Vet Intern Med.* 2014;28:160-165.

12. Midence JN, Drobatz KJ, Hess RS. Cortisol concentrations in well regulated dogs with hyperadrenocorticism treated with trilostane. *J Vet Intern Med.* 2015;29:1529-1533.

13. McGrotty YL, Arteaga A, Knottenbelt CM, et al. Haptoglobin concentrations in dogs undergoing trilostane treatment for hyperadrenocorticism. *Vet Clin Pathol*. 2005;34:255-258.

14. Galac S, Buijtels JJ, Kooistra HS. Urinary corticoid:creatinine ratios in dogs with pituitary-dependent hyperadrenocorticism during trilostane treatment. *J Vet Intern Med.* 2009;23:1214-1219.

15. Arteaga A, Dhand NK, McCann T, et al. Monitoring the response of canine hyperadrenocorticism to trilostane treatment by assessment of acute phase protein concentrations. *J Small Anim Pract.* 2010;51: 204-207.

16. Cook AK, Bond KG. Evaluation of the use of baseline cortisol concentration as a monitoring tool for dogs receiving trilostane as a treatment for hyperadrenocorticism. *J Am Vet Med Assoc*. 2010;237: 801-805.

17. Burkhardt WA, Boretti FS, Reusch CE, Sieber-Ruckstuhl NS. Evaluation of baseline cortisol, endogenous ACTH, and cortisol/ACTH ratio to monitor trilostane treatment in dogs with pituitary-dependent hyperadrenocorticism. *J Vet Intern Med.* 2013;27:919-923.

18. Ramsey IK, Sieber-Ruckstuhl N & Woods G et al. Haptoglobin and pre-trilostane cortisol as monitoring tools for the treatment of canine hyperadrenocorticism. 28th ECVIM-CA congress; Rotterdam, The Netherland; 2018.

19. Boretti F, Musella C, Burkhardt WA, et al. Comparison of two prepill cortisol concentrations in dogs with hypercortisolism treated with trilostane. *BMC Vet Res.* 2018;14:1-7.

20. Martinez-Subiela S, Cer on JJ, Ginel PJ. Effects of different glucocorticoid treatments on serum acute phase proteins in dogs. *Vet Rec*.2004;154:814-817.

21. Harvey JW, West CL. Prednisone-induced increases in serum alpha-2-globulin and haptoglobin concentrations in dogs. *Vet Pathol.* 1987;24:90-92.

22. McGrotty YL, Knottenbelt CM, Ramsey IK, et al. Haptoglobin concentrations in a canine hospital population. *Vet Rec.* 2003;152:562-564.

23. Behrend EN, Kooistra HS, Nelson R, Reusch CE, Scott-Moncrieff JC. Diagnosis of spontaneous canine hyperadrenocorticism: 2012 ACVIM consensus statement (small animal). *J Vet Intern Med.* 2013;27:1292-1304.

24. Kooistra HS, Voorhout G, Mol JA, Rijnberk A. Correlation between impairment of glucocorticoid feedback and the size of the pituitary gland in dogs with pituitary-dependent hyperadrenocorticism. *J Endocrinol.* 1997;152:387-394.

25. Barthez PY, Nyland TG, Feldman EC. Ultrasonographic evaluation of the adrenal glands in dogs. *J Am Vet Med Assoc*. 1995;207:1180-1183.

26. Feldman EC, Nelson RW, Feldman MS. Use of low-and high-dose dexamethasone tests for distinguishing pituitary-dependent from adrenal tumor hyperadrenocorticism in dogs. *J Am Vet Med Assoc*. 1996;209:772-775.

27. Van Liew CH, Greco DS, Salman MD. Comparison of results of adrenocorticotropic hormone stimulation and low-dose dexamethasone suppression tests with necropsy findings in dogs: 81 cases (1985-1995). *J Am Vet Med Assoc.* 1997;211:322-325.

28. Gould SM, Baines EA, Mannion PA, Evans H, Herrtage ME. Use of endogenous ACTH concentration and adrenal ultrasonography to distinguish the cause of canine hyperadrenocorticism. *J Small Anim Pract.* 2001;42:113-121.

29. Pérez-Alenza D, Melián C. Hyperadrenocorticism in dogs. In: Ettinger SJ, Feldman EC, Côté E, eds. Textbook of Veterinary Internal Medicine. 8th ed. St. Louis, MO: Elsevier; 2017:1795-1811.

30. van Bokhorst KL, Kooistra HS, Boroffka SAEB, Galac S. Concurrent pituitary and adrenocortical lesions on computed tomography imaging in dogs with spontaneous hypercortisolism. *J Vet InternMed.* 2018;33:72-78.

31. Wu ZQ, Xu HG. Preanalytical stability of adrenocorticotropic hormone depends on both time to centrifugation and temperature. *J Clin Lab Anal*. 2017;31:22081.

32. Singh AK, Jiang Y, White T, Spassova D. Validation of nonradioactive chemiluminescent immunoassay methods for the analysis of thyroxine and cortisol in blood samples obtained from dogs, cats, and horses. *J Vet Diagn Invest.* 1997;9:261-268.

33. Scott-Moncrieff JC, Koshko MA, Brown JA, Hill K, Refsal KR. Validation of a chemiluminescent enzyme immunometric assay for plasma adrenocorticotropic hormone in the dog. *Vet Clin Pathol*. 2003;32:180-187.

34. Mastrorilli C, Dondi F, Agnoli C, Turba ME, Vezzali E, Gentilini F. Clinicopathologic features and outcome predictors of Leptospira interrogans Australis serogroup infection in dogs: a retrospective study of 20 cases. *J Vet Intern Med*. 2007;21:3-10.

35. Cuzzola A, Mazzini F, Petri A. A comprehensive study for the validation of a LC–MS/MS method for the determination of free and total forms of urinary cortisol and its metabolites. *J Pharm Biomed Anal.* 2014;94:203-209.

36. Pekkin AM, Hänninen L, Tiira K, et al. The effect of a pressure vest on the behaviour, salivary cortisol and urine oxytocin of noise phobic dogs in a controlled test. *Appl Anim Behav Sci.* 2016;185:86-94.

37. Derezi nski P, Klupczy nska A, Sawicki W, Kokot ZJ. Creatinine determination in urine by liquid chromatography-electrospray uonizationtandem mass spectrometry method. *Acta Pol Pharm.* 2016;73:303-313.

38. Baldo FD, Magna L, Dondi F, et al. Comparison of serum fructosamine and glycated hemoglobin values ìfor assessment of glycemic control in dogs with diabetes mellitus. *Am J Vet Res*. 2020;81:233-242.

39. Zeugswetter FK, Neffe F, Schwendenwein I, Tichy A, Möstl E. Configuration of antibodies for assay of urinary cortisol in dogs influences analytic specificity. *Domest Anim Endocrinol.* 2013;45:98-104.

40. Witt AL, Neiger R. Adrenocorticotropic hormone levels in dogs with pituitary-dependent hyperadrenocorticism following trilostane therapy. *Vet Rec.* 2004;154:399-400.

41. Sieber-Ruckstuhl NS, Boretti FS, Wenger M, Maser-Gluth C, Reusch CE. Cortisol, aldosterone, cortisol precursor, androgen and endogenous ACTH concentrations in dogs with pituitary-dependant hyperadrenocorticism treated with trilostane. *Domest Anim Endocrinol*. 2006;31:63-75.

42. Cuhadar S, Atay A, Koseoglu M, et al. Stability studies of common biochemical analytes in serum separator tubes with or without gel barrier subjected to various storage conditions. *Biochem Med.* 2012; 22:202-214.

43. Schouwers S, Brandt I, Willemse J, et al. Influence of separator gel in Sarstedt S-Monovette® serum tubes on various therapeutic drugs, hormones, and proteins. *Clin Chim Acta***.** 2012;413:100-104.

44. Sanders K, Kooistra HS, Galac S. Treating canine Cushing's syndrome: current options and future prospects. *Vet J*. 2018;241:42-51.

Chapter 7

Evaluation of clinical, ultrasonographic, and clinicopathological findings in dogs with pituitary-dependent hypercortisolism and poor trilostane response

Stefania Golinelli, Francesca del Baldo, Rodolfo Oliveira Leal,

Federica Alessandrini, Pascaline Pey, Federico Fracassi

Ready for submission J Vet Intern Med.

Dipartimento di Scienze Mediche Veterinarie,

Scuola di Agraria e Medicina Veterinaria,

Bologna

Abstract

Background: Trilostane is effective in about 85-90% of dogs with pituitary-dependent hypercortisolism (PDH). Factors able to predict trilostane efficacy have never been evaluated.

Objectives: To compare different clinical, ultrasonographic, and clinical-pathological variables between dogs with good (GRDs) and poor (PRDs) clinical response to trilostane treatment.

Animals: Thirty-four dogs with PDH treated with trilostane twice daily.

Methods: Retrospective study. Signalment, history, therapeutic responses, clinicopathologic and diagnostic imaging results were recorded from PDH dogs treated with trilostane. PRDs were identified if ≥ 2 PDH signs persisted after a 6-month follow-up, despite a trilostane dose ≥ 3 mg/kg q12h.

Results: GRDs (n=24) exhibited significantly lower serum ALT (*P*=.004), γGT (*P*=.039), phosphate (*P*=.039), eACTH (*P*=.027), pre-ACTH cortisol (*P*=.004), and 8h-post dexamethasone cortisol concentrations (*P*=.019) at diagnosis compared to PRDs (n=10). Additionally, GRDs had lower creatinine levels (*P*=.006) and higher trilostane starting doses (*P*=.021). A higher proportion of GRDs lacked bilateral adrenomegaly (*P*=0.003), and alopecia at diagnosis was significantly more prevalent in PRDs (*P*=0.015).

Six months post-treatment, GRDs demonstrated significant reductions in ALT (*P*<.001), γGT (*P*=0.005), ALP (*P*=0.005), cholesterol (*P*<.001), phosphate (*P*=0.022), pre-ACTH cortisol (*P*<.001), post-ACTH cortisol (*P*<.001) with higher creatinine levels (*P*=0.003). Alopecia, specific clinicopathological variables, cortisol concentrations, bilateral adrenomegaly, and trilostane dose at diagnosis were associated with a poor response to trilostane.

Conclusions and Clinical Importance: Alopecia, specific clinicopathological variables, cortisol concentrations, bilateral adrenomegaly, and trilostane dose at diagnosis were associated with a poor response to trilostane. These findings contribute to understanding treatment outcomes and may guide personalized therapeutic approaches in veterinary practice.

Introduction

Pituitary-dependent hypercortisolism (PDH) accounts for 80-85% of all cases of spontaneous canine hypercortisolism (HC).[1] Trilostane has emerged as the preferred medical treatment for canine PDH over the past two decades. Functioning as a competitive inhibitor of the 3b-hydroxysteroid dehydrogenase/isomerase system, a pivotal enzyme system in cortisol, aldosterone, and androstenedione synthesis, trilostane has shown efficacy in significantly reducing basal and ACTH-stimulated plasma cortisol concentrations in dogs with PDH.[2–5] This leads to a loss in negative feedback, resulting in an elevation of endogenous plasma ACTH (eACTH) concentration.[6–8]

The optimal objectives in treating canine hypercortisolism involve resolving clinical signs, mitigating prolonged complications and mortality risks, improving the overall quality of life, and ideally, eliminating the source of excessive ACTH or autonomous cortisol production.[9] Recent studies have demonstrated that trilostane can successfully achieve these goals, with the exception of eradicating the underlying source.[10-18]

However, the gradual and variable control of clinical signs has been reported in studies, with good control ranging from less than 50% to 100% of treated dogs within a few weeks.[3,8,11-17] After several months of treatment, partial to complete control of clinical signs occurred in over 75% of cases in published studies.[3,8,11-17] Adverse effects, generally mild to moderate, were reported in 0% to 40% of cases.[3,8,11-17]

Assessment of trilostane treatment effectiveness involves the resolution of clinical signs associated with glucocorticoid excess and cortisol concentration measurements, typically through an ACTH stimulation test or prepill cortisol.[19] Clinical signs such as polyuria/polydipsia and reduced activity usually resolve sooner, while dermatological abnormalities may take up to 6 months.[12]

The frequency of trilostane administration, often suggested to be every 12 hours, can influence drug efficacy.[10,12-17] The final dose of trilostane for achieving partial to complete resolution of clinical signs reported in studies published in the last 15 years ranges between .21 to 4.45 mg/kg q12h.[12-14,16,17] Approximately 10-15% of dogs treated with trilostane, despite the increase in the dose, do not exhibit improvement in the clinical picture and clinicopathological variables.[3,8,11-17] Factors predicting trilostane efficacy have not been reported in the veterinary literature. Understanding the likelihood of a dog responding to the drug's action could be useful for veterinarians and owners in planning the PDH treatment. This retrospective study aims to compare several clinical, ultrasonographic, and clinicopathological findings between dogs with a favorable clinical response and those demonstrating a poor response to trilostane treatment.

Materials and Methods

A retrospective cohort study in dogs was performed in two European Veterinary Teaching Hospitals.

Dogs

Dogs were identified through an electronic medical record search. Dogs were included if, at the time of diagnosis, exhibited a minimum of two of the following clinical signs indicative of HC: polyuria and polydipsia (PU/PD) (considered as a single clinical sign), polyphagia, alopecia, and/or abdominal enlargement and at least one abnormal screening test result consistent with HC. The screening tests considered were low-dose dexamethasone suppression test (LDDSt) and/or adrenocorticotropic hormone stimulation test. (ACTHst).[1] If a dog underwent also urine corticoid: creatinine ratio (UCCR), this was recorded. Only dogs diagnosed with PDH were considered in the study.

The localization of HC at a pituitary level (PDH) was based on the result of the LDDSt[1] and/or reference range-to-increased eACTH[1] concentration, and/or bilateral normal-to-increased adrenal gland size observed via abdominal ultrasonography, and/or evidence of a pituitary mass indicated by diagnostic imaging techniques (magnetic resonance imaging [MRI] or computed tomography [CT]).[1]

Additionally, dogs were required to undergo a follow-up of trilostane (Vetoryl, Dechra, Shrewsbury, UK) treatment for 6 months. Only dogs treated with trilostane twice daily were included. Dogs could have initially been treated with trilostane every 24 hours (q24h) but the frequency of treatment had to be transitioned to twice daily within the first check. Collected data for each dog at diagnosis encompassed signalment details (age, breed, gender, neuter status, and body weight), medical history, clinical findings (PU/PD, polyphagia, abdominal enlargement, and alopecia), systolic arterial blood pressure (SAP), presence of concurrent disorders, clinicopathological variables (serum alanine aminotransferase [ALT], alkaline phosphatase [ALP], gamma-glutamyl transferase [γGT], cholesterol, creatinine, urea, phosphate, urine specific gravity [USG], and urine protein-to-creatinine ratio [UPC]), results of endocrine tests (pre-ACTH cortisol [pre-ACTH], post-ACTH cortisol [post-ACTH], T4 and T8, UCCR and eACTH concentrations), diagnostic imaging findings (CT/MRI), and adrenal ultrasound results (increased in volume or normal adrenal glands). Adrenal glands were considered to be increased in volume according to dog size.[20] Pituitary gland was considered as macroadenoma if the pituitary: brain (P/B) ratio was above .31.[21]

Dogs demonstrating a complete resolution of clinical signs, including the absence of PU/PD, polyphagia, abdominal enlargement, and alopecia at the 6-month follow-up, were categorized as good responder dogs (GRDs). Conversely, poor responder dogs (PRDs) were identified when one or more clinical signs persisted at the 6-month follow-up, accompanied by a trilostane dose exceeding 3 mg/kg q12h.

Monitoring trilostane treatment involved assessing clinical signs, blood and urine examination results, and cortisol concentrations (pre-trilostane administration cortisol [prepill] or ACTHst). These parameters were collected for each dog participating in the study during the 6-months follow-up. Elevated concentrations of prepill and post-ACTH cortisol exceeding 5 μg/kg were indicative of inadequate control of trilostane treatment.[22]

All the biochemical variables were measured using an automatic analyzer (AU480, Beckman Coulter/Olympus, Brea, California) while serum and urine cortisol and plasma eACTH were measured using a chemiluminescent enzyme immunoassay (Immulite 2000, Siemens Healthcare).[23,24] Trilostane treatment details, such as starting and final doses at the 6-months follow-up, starting frequency of administration, and therapy responses, were also recorded for each case.

Exclusion criteria mandated the absence of owner-reported concerns such as vomiting, diarrhea, or poor appetite and the absence of a trilostane treatment follow-up of 6 months.

Data Analysis

Collected data were managed with an electronic spreadsheet (Microsoft Excel) and analyzed using commercial statistical data analysis software (Prism7.0a, GraphPad Software, Inc., San Diego, California). The D'Agostino and Pearson test was used to assess the normality of continuous data. General and clinical characteristics of dogs with PDH included in the study were summarized using median and range. Statistical comparisons between the two groups were conducted using the Mann-Whitney test for continuous variables and the Chi-square test for categorical variables. For variables found to be significant in the Mann-Whitney test, Receiver Operating Characteristic (ROC) curves were generated to assess diagnostic accuracy. Sensitivity, specificity, likelihood ratios, and cutoff values were calculated based on these ROC curves. The cutoff value selected for both sensitivity and specificity was determined by identifying the point on the

ROC curve with the highest likelihood ratio. This approach aimed to optimize specificity, minimizing false positives and avoiding the immediate exposure of potentially responsive dogs to more intensive and costly treatments. The chosen cutoff aimed for a balance between specificity and sensitivity, ensuring a higher likelihood of correctly identifying non-responsive cases while accepting a lower sensitivity to avoid unnecessary and costly alternative treatments in potentially trilostane-responsive cases. Regarding the biochemistry and urinalysis clinicopathological parameters, only ALT, ALP, GGT, cholesterol, creatinine, urea, phosphorus, and UPC that were measured utilizing the same analytical method at the Veterinary Teaching Hospital of the University of Bologna were considered in the comparative analysis. A p-value of <.05 was considered significant.

Results

Thirty-four dogs with PDH treated with trilostane for 6 months met the inclusion criteria, with 29 dogs from the Veterinary Teaching Hospital of the University of Bologna and 5 dogs from the Veterinary Teaching Hospital of the University of Lisbon between June 2013 and March 2023.

Good Responder Dogs (GRDs)

Twenty-four out of 34 dogs (71%) included, 19 from the University of Bologna and 5 from the University of Lisbon, were classified as GRDs. Among them, 8 were male (4 intact and 4 neutered), and 16 were female (1 intact and 15 neutered). Breeds included 9 mixed breeds, 3 Yorkshire Terrier, 3 Beagle, 2 Dachshunds, and 1 each Poodle, Border Terrier, Weimaraner, Jack Russel Terrier, Lagotto, Fox Terrier, Pinscher, and Labrador Retriever. The median body weight was 12 kg (3.6-44.5), and the median age at PDH diagnosis was 11 years (4-15). The starting trilostane dose was .84 mg/kg (.45-1.5) daily, with an initial frequency of administration every 24h in 3 out of 24 GRDs. At diagnosis, 22/24 showed PU/PD, 19/24 polyphagia, 10/24 abdominal enlargement, and 14/24 alopecia as clinical signs.

Diagnosis of PDH was confirmed in 17/24 dogs with LDDSt results and in 17/24 dogs with ACTHst results. In 7/24 dogs UCCR was also evaluated. Ten out 24 GRDs had two diagnostic tests performed, and 3 out 24 dogs had all 3 diagnostic tests performed. Testing to discriminate PDH from adrenal-dependent hypercortisolism (ADH) included eACTH measurements (18/24 GRDs), abdominal ultrasonography (20/24 GRDs), and pituitary CT (9/24 GRDs). Adrenal gland resulted enlarged in 9 out 20 GRDs and normal in 11/20 dogs. Seven out 9 dogs that underwent pituitary CT had a microadenoma and 2/9 had a macroadenoma. Median SAP was 160 mmHg (160-196).

Concurrent conditions/diseases were present at diagnosis in 11 out of 24 GRDs: biliary sludge (5), otitis (2), arthrosis (1), hepatic tumor (1), 1 myxomatous valve disease (1), mucocele (1), food responsive enteropathy (1), and cognitive dysfunction syndrome (1).

At the 6-months follow-up, the median trilostane dose was 1.06 mg/kg (.45-2.5) q12h, with monitoring performed through ACTHst (11/24 GRDs), prepill cortisol (10/24 GRDs), or both (3/24 GRDs). Post-ACTH and prepill cortisol concentrations were above 5 µg/kg in 6/24 GRDs (25%) (3 had prepill > 5 µg/kg and 3 had post-ACTH > 5 µg/kg). The following concurrent conditions/diseases were present at the 6-month follow-up in 12/24 GRDs: biliary sludge (5), otitis (2), arthrosis (1), hepatic tumor (1), myxomatous valve disease (1), mucocele(1), food responsive enteropathy(1), and 1 cognitive dysfunction syndrome(1).

Poor Responder Dogs (PRDs)

Ten out 34 dogs (29%) included in the study, all from the University of Bologna, were classified as PRDs. Among them,

6 were male (4 intact and 2 neutered), and 4 were female (2 intact and 2 neutered). Breeds included 5 mixed-breed, 2 Maltese, and 1 each Yorkshire Terrier, Shih-tzu, and French Bouledogue. The median body weight was 8.3 kg (3.5-15.6), and the median age at PDH diagnosis was 8 years (6-13). The starting trilostane dose was 1.22 mg/kg (.7-2) daily, administered every 24h in 1 out of 10 PRDs. At diagnosis 8/10 PRDs showed PU/PD, 9/10 PRDs polyphagia, 6/10 PRDs abdominal enlargement, and 10/10 PRDs alopecia as clinical signs.

Diagnosis of PDH was confirmed in 4/10 dogs with LDDSt results and in 6/10 dogs with ACTHst results. In 3/10 PRDs UCCR was also evaluated. Two out 10 PRDs had 2 diagnostic tests performed, 1 out 10 dogs had all 3 diagnostic tests performed. Testing to discriminate PDH from ADH included eACTH measurements (10/10 PRDs), abdominal ultrasonography (10/10 PRDs), and pituitary CT (4/10 PRDs). Adrenal glands were enlarged in all 10 PRDs. Two out 4 dogs that underwent pituitary CT had a microadenoma and 2/4 had a macroadenoma. Median SAP was 152 mmHg (111-210).

Concurrent conditions/diseases were present at diagnosis in 6 out of 10 PRDs: biliary sludge (2), mucocele (1), arthrosis (1), urinary tract infection (1), urolithiasis (1), myxomatous valve disease (1), mammary tumors (1), and hypothyroidism (1). At the 6-month follow-up, the median trilostane dose was 3.25 mg/kg (3-7) q12h, with monitoring performed through ACTHst (3/10 PRDs), prepill cortisol (4/10 PRDs), both (2/10 PRDs) and in one dog with only the clinical signs. Prepill cortisol concentrations were below 5 µg/kg in 1/10 PRDs (10%). The following concurrent conditions/diseases were present at the 6-month follow-up in 7/10 PRDs: mucocele (3), arthrosis (2), urinary tract infection (1), urolithiasis (1), myxomatous valve disease (1), mammary tumors (1), and hypothyroidism (1).

Comparison of GRDs and PRDs

The median and range of GDRs and PRDs continuous variables at diagnosis and 6-months follow-up are reported in Table 1 and Table 2, respectively.

Table 1. Median and range of GRDs and PRDs continuous variables at diagnosis

	GRDs (n = 24)				PRDs (n =10)				*P* value
	n	Median	Min.	Max.	n	Median	Min.	Max.	
ALT (U/L)	19	164	40	442	10	311	74	962	**.004**
ALP (U/L)	19	459	46	14075	10	1455	160	17347	.057
GGT (U/L)	17	5.06	2.76	7.30	10	5.81	4.58	7.23	**.039**
Cholesterol (mg/dl)	17	419	271	955	10	582	222	906	.204
Creatinine (mg/dl)	19	.73	.41	1.34	10	.54	.30	0.67	**.006**
Urea (mg/dl)	19	27	9	77	10	20	7	42	.060
USG (mg/dl)	23	1015	1004	1038	10	1014	1006	1038	.992
UPC	17	1.4	.1	10.4	9	1.8	.3	17.3	.569
Phosphorus	17	5.06	2.76	7.30	9	5.81	4.58	7.23	**.039**
Pre-ACTH (µg/dl)	17	4.2	2.4	9.2	6	8.9	5.1	28	**.004**
Post-ACTH (µg/dl)	17	30	18	51	6	50	8.6	50	.101
T4 (µg/dl)	17	1.5	.5	11.6	4	2.7	1	10.7	.144
T8 (µg/dl)	17	1.9	.5	8.2	4	5.8	3.7	6.8	**.019**
UCCR	7	93	35	192	3	110	82	505	.383
eACTH (pg/ml)	18	14.5	5	68.4	10	47.9	10.4	128	**.027**
Age (years)	24	11	4	15	10	8	6	13	.077
Weight (Kg)	24	12	3.6	44.5	10	8.3	3.5	15.6	.180
Trilostane Starting Dose (mg/dl)	24	.84	.45	1.50	10	1.22	.70	2	**.021**
SAP (mmHg)	15	159	110	200	7	163	108	190	.848

Abbreviation: GRDs, good responder dogs; PRDs, poor responder dogs, ALT, alanine aminotransferase; ALP; alkaline phosphatase; γGT, gamma-glutamyl transferase; pre-ACTH, pre-ACTH cortisol; post-ACTH, post-ACTH cortisol; T4, 4h post-dexamethasone cortisol; T8, 8h post-dexamethasone cortisol; UCCR, urine cortisol-to-creatinine ratio; eACTH, endogenous ACTH; SAP, systolic arterial blood pressure.

Table 2. Median and range of GRDs and PRDs continuous variables at 6 months follow-up

	GRDs (n = 24)				PRDs (n =10)				*P* value
	n	Median	Min.	Max.	n	Median	Min.	Max.	
ALT (U/L)	19	26	40	881	10	239	73	634	**< .001**
ALP (U/L)	19	308	28	8979	10	811	333	12325	**.005**
GGT (U/L)	11	1.9	2.76	12.3	7	46	4.3	192	**.005**
Cholesterol (mg/dl)	18	307	156	458	10	503	314	788	**< .001**
Creatinine (mg/dl)	19	.8	.51	1.27	10	.51	.4	1.11	**.003**
Urea (mg/dl)	19	33	13	72	10	27	14	64	.224
USG (mg/dl)	18	1019	1006	1052	7	1022	1004	1025	.965
UPC	15	1.2	.07	4.7	7	2.4	.18	16.5	.244
Phosphorus	19	4.37	2.87	5.96	10	5.64	3.9	7.62	**.022**
Pre-ACTH (μg/dl)	14	1.13	0.92	2.71	5	5.90	4.79	10	**< .001**
Post-ACTH (μg/dl)	17	3.43	.74	8.76	6	8.9	8.64	11	**< .001**
Prepill	13	3.0	1.10	6.9	6	7.45	1.75	15.4	.058
Trilostane Dose (mg/dl)	24	1.06	.45	2.5	10	3.25	3	7	**< .001**
SAP (mmHg)	11	160	140	196	9	152	111	210	.955

Abbreviation: GRDs, good responder dogs; PRDs, poor responder dogs, ALT, alanine aminotransferase; ALP; alkaline phosphatase; γGT, gamma-glutamyl transferase; pre-ACTH, pre-ACTH cortisol; post-ACTH, post-ACTH cortisol; prepill, prepill cortisol; SAP, systolic arterial blood pressure.

At diagnosis, serum ALT (P = .004), γGT (P = .039), and phosphate (P = .039) were significantly higher in PRDs compared to GRDs. Additionally, pre-ACTH (P = .004), eACTH (P = .027), T8 (P = .019), and trilostane starting dose (P = .021) were higher in PRDs, while creatinine was significantly lower in PRDs (P = .006) (Figure 1).

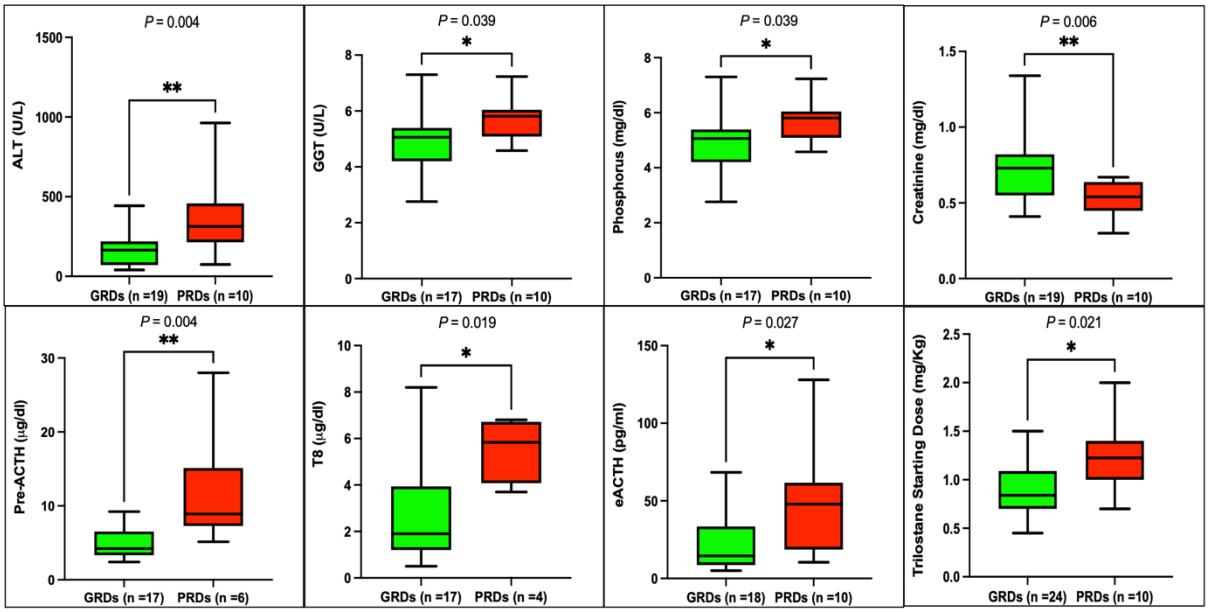

Figure 1 Box and whisker plots of ALT, GGT, phosphorus, creatinine, pre-ACTH, T8, eACTH concentrations, and trilostane starting dose at PDH diagnosis divided into two groups according to trilostane treatment response, the GRDs and PRDs. The lower and upper boundaries of the box represent the first and third quartiles of the data, respectively, with the line within the box representing the median. The whiskers represent the 5th to 95th percentile. Significantly different results are indicated by connecting horizontal lines with the P values shown above. ALT, alanine aminotransferase; γGT, gamma-glutamyl transferase; pre-ACTH, pre-ACTH cortisol; T8, 8h post-dexamethasone cortisol; eACTH, endogenous ACTH; GRDs, good responder dogs; PRDs, poor responder dogs; PDH, pituitary-dependent hypercortisolism.

Results of ROC curve analysis, specificity, sensitivity, and Likelihood ratio at diagnosis are reported in Table 3.

Table 3. ROC curve analysis results

	AUC	Specificity %	Sensitivity %	Cutoff	Likelihood Ratio
ALT (U/L)	.82	60	89	>278	5.70
GGT (U/L)	.74	60	82	>5.6	3.4
Creatinine (mg/dl)	.81	30	95	<.46	5.7
Phosphorus	.74	60	82	>5.6	3.4
Pre-ACTH (μg/dl)	.88	67	94.2	>8.7	11.3
T8 (μg/dl)	.87	50	94	>6.3	8.5
eACTH (pg/ml)	.76	30	94	>60.3	5.4
Trilostane Starting Dose (mg/dl)	.75	60	79	>1.16	2.9

The AUC, cutoff value, specificity, sensitivity, and accuracy of each variable significantly different between GRDs and PRDs are reported.
Abbreviation: ALT, alanine aminotransferase; ALP; alkaline phosphatase; γGT, gamma-glutamyl transferase; pre-ACTH, pre-ACTH cortisol; T8, 8h post-dexamethasone; eACTH, endogenous ACTH, GRDs, good responder dogs; PRDs, poor responder dogs.

Chi-square results are reported in Table 4, showing a higher percentage of adrenomegaly (P = .003) and alopecia (P = .015) at the time of diagnosis in PRDs.

Table 4. Chi-square results at diagnosis

	GRDs				PRDs				P value	Chi-square (X^2)
Sex	4/24 (16.7%) M	4/24 (16.7%) MN	1 (4.2%) F	15 (62.5 %) FN	4/10 (40%) M	2/10 (20%) MN	2/10 (20%) F	2/10 (20%) FS	**< .001**	**6.23**
US Adrenal Size	9/20 (45%) Adrenomegaly		11/20 (55%) Normal Adrenals		10/10 (100%) Adrenomegaly		0/10 (0%) Normal Adrenals		.003	8.68
Frequency of Administration	3/24 (12.5%) SID		21/24 (87.5%) BID		1/10 (10%) SID		9/10 (90%) BID		.837	.04
Concurrent Diseases	11/24 (45.8%) Yes		13/24% (54.2%) No		6/10 (60%) Yes		4/10 (402%) No		.452	.057
PU/PD	22/24 (91.7%) Yes		2/24 (8.3%) No		8/10 (80%) Yes		2/10 (20%) No		.336	.926
Polyphagia	19/24 (79.2%) Yes		5/24 (20.8%). No		9/10 (90%) Yes		1/10 (10%) No		.450	.570
Abdominal Enlargement	10/24 (41.7%) Yes		14/24 (58.3%). No		6/10 (60%) Yes		4/10 (40%) No		.329	.952
Alopecia	14/24 (58.3%) Yes		10/24 (41.7) No		10/10 (100%) Yes		0/10 (0%) No		**.015**	**5.90**

Abbreviation: GRDs, good responsive dogs; PRDs, poor responsive dogs; M, intact male, MC, neutered male; F, intact female; FN, neutered female; UD, ultrasound; PU/PD, polyuria/polydipsia.

At the 6-month follow-up, several parameters including serum ALT (P < .001), ALP (P = .005), γGT (P = .005), cholesterol (P < .001), phosphate (P = .022), pre-ACTH (P < .001), post-ACTH (P < .001), and trilostane dose (P < .001) were significantly higher in PRDs compared to GRDs, while creatinine was significantly lower in PRDs (P = .003) (Figure 2).

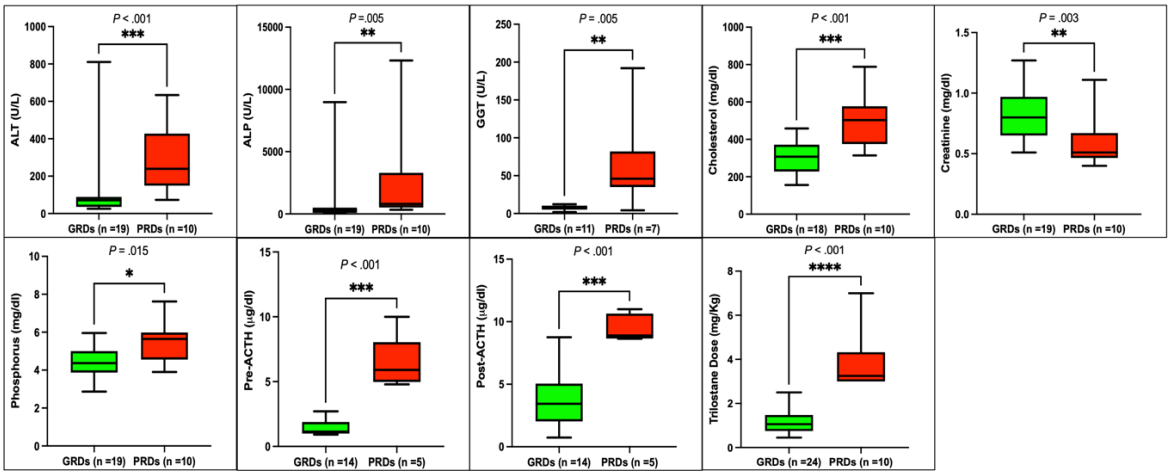

Figure 2 Box and whisker plots of ALT, ALP, GGT, cholesterol, phosphorus, creatinine, pre-ACTH, post-ACTH concentrations, and trilostane dose at the 6 months follow-up divided into two groups according to trilostane treatment response, the GRDs and PRDs. The lower and upper boundaries of the box represent the first and third quartiles of the data, respectively, with the line within the box representing the median. The whiskers represent the 5th to 95th percentile. Significantly different results are indicated by connecting horizontal lines with the P values shown above. ALT, alanine aminotransferase; ALP; alkaline phosphatase; γGT, gamma-glutamyl transferase; pre-ACTH, pre-ACTH cortisol; post-ACTH, post-ACTH cortisol; GRDs, good responder dogs; PRDs, poor responder dogs.

Chi-square results at the 6-month follow-up revealed significant differences in the prevalence of PU/PD, polyphagia, abdominal enlargement, and alopecia between the two groups (P < .001) (Table 5).

Table 5. Chi-square results at 6 months follow-up

	GRDs		PRDs		*P* value	*Chi-square* (X^2)
Concurrent Diseases	12/24 (50%) Yes	12/24% (50%) No	7/10 (70%) Yes	3/10% (30%) No	0.594	.283
PU/PD	0/24 (0%) Yes	24/24 (100%) No	6/10 (60%) Yes	4/10 (40%) No	**< .001**	**17.49**
Polyphagia	0/24 (0%) Yes	24/24 (100%) No	6/10 (60%) Yes	4/10 (40%) No	**< .001**	**17.49**
Abdominal Enlargement	0/24 (0%) Yes	124/24 (100%) No	6/10 (60%) Yes	4/10 (40%) No	**< .001**	**17.49**
Alopecia	0/24 (0%) Yes	24/24 (100%) No	10/10 (100%) Yes	0/10 (0%) No	**< .001**	**34**

Abbreviation: GRDs, good responsive dogs; PRDs, poor responsive dogs; PU/PD, polyuria/polydipsia.

Discussion

This study evaluated dogs exhibiting differential responses to trilostane treatment, specifically comparing those with a positive response to those with persistent clinical signs. Trilostane stands as the primary medical intervention for canine PDH; however, existing literature reports approximately 10-15% of dogs as "resistant" to this medication.[3,8,11-17] Our study revealed a higher percentage of trilostane-resistant dogs compared to previously published data, with 29% of the subjects classified as poor responders. Two factors may have influenced this elevated proportion of poor responders: 1) The recruitment of dogs occurred at veterinary teaching hospitals serving as reference centers. It is plausible that cases where the referring veterinarian observed a poor response to treatment might have been redirected to these reference centers, whereas cases with a positive response were more likely to remain under the care of the referring veterinarians. This potential bias in recruitment could impact the overall composition of the study population. 2) The criteria for defining poor responders were limited to dogs showing no clinical signs of PDH at 6 months. This stringent definition implies a

complete clinical resolution of PDH after 6 months of trilostane treatment. While the majority of dogs experience a resolution of clinical signs within this timeframe, certain dermatological abnormalities may persist for a longer duration. Our study identified various clinical, clinicopathological, and ultrasonographic variables associated with a poor response to trilostane treatment in dogs with PDH. Poor responders exhibited significantly elevated concentrations of serum ALT, γGT, phosphate, pre-ACTH cortisol, T8 cortisol, and eACTH, along with an increased trilostane starting dose. Conversely, dogs with a positive response showed higher concentrations of creatinine. Additionally, alopecia and bilateral adrenomegaly were significantly correlated with a poor response to trilostane treatment. Considering these results, in general dogs with a more pronounced clinical picture and more evident classical clinicopathological abnormalities are more likely to respond less to trilostane treatment.

The trilostane starting dose was notably higher in PRDs compared to GRDs. This could be attributed to clinicians being influenced by the severity of clinical and clinicopathological findings, leading them to initiate treatment in more symptomatic patients or those with worse blood exam results with higher doses of trilostane. Over the years the starting dose of trilostane to treat dogs with PDH has been progressively decreased to reduce the episodes of iatrogenic hypocortisolism but still controlling clinical signs.[16] We cannot therefore exclude that the starting dose may have some influence on the clinical response to therapy.

The same clinicopathological variables (ALT, γGT, phosphorus, pre-ACTH cortisol) in addition to ALP, cholesterol, and post-ACTH cortisol concentrations, were significantly higher in PRDs at the 6-month follow-up compared to GRDs. Similarly, creatinine concentration was significantly lower in PRDs when compared with GRDs after 6 months of trilostane treatment. These parameters are generally associated with the hypercortisolemic state over a 24-hours period. It is, therefore, not surprising that they were significantly higher in subjects with poor clinical control of the pathology. In all PRDs, except one, the concentrations of prepill or post-ACTH cortisol exceeded the cutoff indicative of good control. Unfortunately, we were unable to determine how cortisol levels vary over the 24 hours of trilostane treatment, but it is highly probable that even in patients clinically not controlled but with well-controlled cortisol levels, fluctuations may occur, leading to higher values of these parameters.

Within the PRDs, 6 dogs had either prepill or post-ACTH cortisol above the cutoff indicative of good trilostane treatment control. This confirms that clinical evaluation remains a cornerstone in monitoring trilostane treatment. Additionally, at the 6-month follow-up, pre- and post-ACTH cortisol levels were significantly higher in PRDs compared to GRDs, aligning with expectations for dogs with a more severe clinical presentation. However, this result was not consistent for the prepill cortisol, deviating from previous studies where the clinical picture correlated significantly with prepill cortisol rather than post-ACTH cortisol.[19,24-26]

The trilostane dose at 6 months was significantly higher in PRDs than in GRDs; this was expected since this was our criteria to differentiate the two groups. We defined poor responder dogs as those patients displaying two or more persistent clinical signs of PDH after 6 months, despite a drug dosage exceeding 3 mg/Kg twice daily. On the contrary, we did not establish a trilostane dose cutoff for dogs with a good response to trilostane treatment as inclusion criteria. Nevertheless, none of the GRDs received more than 3 mg/Kg q12h of trilostane. This dosage cutoff was arbitrarily determined based on our experience but we also considered the recent literature.[12-14,16,17] Recent studies with only twice-daily administration report much lower final trilostane doses compared to older studies.[3,8,12-14,16,17] Typically, in our institution, we propose to the owner alternative treatments (e.g., mitotane or hypophysectomy) when, despite escalating the dosage, dogs show no improvement in clinical signs, and the dose exceeds 3 mg/Kg q12h.

The subjective nature of deciding the cutoff dosage to define dogs with a poor response may be a limitation of the present study, as it could introduce potential recruiting bias by including poor responder dogs that might respond to higher doses

of trilostane. However, trilostane is not without risks, and one of the treatment goals for PDH is to avoid iatrogenic hypocortisolism. This consideration influenced our choice of cutoff to prevent potential adverse effects.[27-30]

We opted to classify dogs as good or poor responders based solely on the clinical picture, not considering clinicopathological variables or cortisol concentrations. This decision acknowledges the absence of a gold standard method for monitoring trilostane treatment, with previous studies emphasizing the pivotal role of the clinical picture in assessing treatment progress.[19,24-26,31]

Dogs exhibiting an inadequate response to trilostane treatment demonstrated a higher percentage of US adrenomegaly at diagnosis compared to those with a good response. It is plausible that PRDs may have been afflicted by HC for a longer duration than dogs with a positive response to trilostane treatment. This hypothesis stems from the notion that a normal appearance of the adrenal gland in hypercortisolemic conditions, as observed in ultrasound scans, might be attributable to an initial stage of the disease.[32]

One big limitation of the study is the limited number of included cases, potentially influencing some non-significant results. The limited number of included dogs is attributed to our highly selective inclusion criteria, particularly in the case of PRDs. Many owners, faced with the progressive escalation of trilostane dosage and considering the associated costs, opt to discontinue treatment. Some others request euthanasia, switch veterinarians, or explore alternative therapies even before the completion of the 6-months treatment period. Consequently, recruiting non-responders into our study cohort has proven to be challenging. The limited canine sample size is associated with a reduced number of patients undergoing advanced diagnostic pituitary imaging (CT/MRI). The insufficient availability of CT/MRI data in the canine cohort precludes us from drawing any definitive conclusions regarding the potential for PRDs to exhibit enlarged pituitary dimensions compared to those with a good response to treatment. Finally, certain critical clinical signs, such as calcinosis cutis, which, based on the author's experience, appears to be associated with an inadequate response to trilostane treatment, could not be assessed due to the presence of this clinical manifestation in only one included dog.

In conclusion, dogs with a more severe clinical presentation and worse clinicopathological abnormalities appear more likely to be resistant to trilostane treatment. Higher concentrations of ALT, γGT, phosphorus, pre-ACTH cortisol, T8 cortisol, eACTH, and trilostane starting dose, coupled with lower creatinine concentrations, increase the likelihood of a poor response to therapy. These results pave the way for prospective studies to confirm and further explore the obtained data.

References

1. Behrend EN. Canine hyperadrenocorticism. In: Feldman EC, Nelson RW, Reusch CE, et al., eds. *Canine and Feline Endocrinology*. 4th ed. St. Louis, MO: Elsevier Saunders; 2015:377-451. [Google Scholar]
2. Neiger R, Ramsey I, O'Connor J, et al. Trilostane treatment of 78 dogs with pituitary-dependent hyperadrenocorticism. *Vet Rec*. 2002;150:799–804.
3. Ruckstuhl NS, Nett CS, Reusch CE. Results of clinical examinations, laboratory tests, and ultrasonography in dogs with pituitary-dependent hyperadrenocorticism treated with trilostane. *Am J Vet Res.* 2002;63:506–512.
4. Wenger M, Sieber-Ruckstuhl NS, Muller C, et al. Effect of trilostane on serum concentrations of aldosterone, cortisol, and potassium in dogs with pituitary-dependent hyperadrenocorticism. *Am J Vet Res.* 2004;65:1245–1250.

5. Bell R, Neiger R, McGrotty Y, et al. Study of the effects of once daily doses of trilostane on cortisol concentrations and responsiveness to adrenocorticotropic hormone in hyperadrenocorticoid dogs. *Vet Rec*. 2006;159:277–281.
6. Witt AL, Neiger R. Adrenocorticotropic hormone levels in dogs with pituitary-dependent hyperadrenocorticism following trilostane therapy. *Vet Rec.* 2004;154:399–400.
7. Sieber-Ruckstuhl NS, Boretti MS, Wenger M, et al. Cortisol, aldosterone, cortisol precursor, androgen and endogenous ACTH concentrations in dogs with pituitary-dependant hyperadrenocorticism treated with trilostane. *Domest Anim Endocrinol*. 2006; 31:63–75.
8. Galac S, Buijtels JJCWM, Mol JA, Kooistra HA. Effects of trilostane treatment on the pituitary-adrenocortical and renin-aldosterone axis in dogs with pituitary-dependent hypercortisolism. *Vet J*. 2008, doi:10.1016/j.tvjl.208.10.007.
9. ALIVE goals of treatment of naturally-occurring Cushing's syndrome. 2021 https://www.esve.org/alive/search.aspx.
10. Sanders K, Kooistra HS, Galac S. Treating canine Cushing's syndrome: current options and future prospects. *Vet J* 2018;241:42-51.
11. Braddock JA, Church DB, Robertson ID, Watson AD. Trilostane treatment in dogs with pituitary-dependent hyperadrenocorticism. *Aust Vet J.* 2003;81:600–607
12. Augusto M, Burden A, Neiger R, Ramsey I. A comparison of once and twice daily administration of trilostane to dogs with hyperadrenocorticism. *Tierarztl Prax K H.* 2012;40:415–424.
13. Arenas C, Melian C, Perez-Alenza MD. Evaluation of 2 trilostane protocols for the treatment of canine pituitary-dependent hyperadrenocorticism: Twice daily versus once daily. *J Vet Intern Med.* 2013;27:1478-1485.
14. Cho KD, Kang JH, Chang D, Na KJ, Yang MP. Efficacy of low-and high-dose trilostane treatment in dogs (< 5 kg) with pituitary-dependent hyperadrenocorticism. *J Vet Intern Med.* 2013;27:91–98.
15. Alenza DP, Arenas C, Lopez ML, Melian C. Long-term efficacy of trilostane administered twice daily in dogs with pituitary-dependent hyperadrenocorticism. *J Am Anim Hosp Assoc.* 2006;42:269–276.
16. Vaughan MA, Feldman EC, Hoar BR, Nelson RW. Evaluation of twice-daily, low-dose trilostane treatment administered orally in dogs with naturally occurring hyperadrenocorticism. *J Am Vet Med Assoc.* 2008;238:1441-1451.
17. Feldman EC. Evaluation of twice-daily lower-dose trilostane treatment administered orally in dogs with naturally occurring hyperadrenocorticism. *J Am Vet Med Assoc.* 2011;238:1441–1451.
18. Nagata, N, Kojima K, Yuki M. Comparison of survival times for dogs with pituitary-dependent hyperadrenocorticism in a primary-care hospital: treated with trilostane versus untreated. *J Vet Intern Med* 2017;31:22-28.
19. Golinelli S, de Marco V, Leal RO, et al. Comparison of methods to monitor dogs with hypercortisolism treated with trilostane. *J Vet Intern Med* 2021;35(6):2616-2627. doi:10.1111/jvim.16269.
20. Soulsby SN, Holland M, Hudson JA, et al. Ultrasonographic evaluation of adrenal gland size compared to body weight in normal dogs. *Vet Radiol Ultrasound*. 2015:56:317-326.
21. Kooistra HS, Voorhout G, Mol JA, et al. Correlation between impairment of glucocorticoid feedback and the size of the pituitary gland in dogs with pituitary-dependent hyperadrenocorticism. *J Endocrinol.* 1997;152:387-394.

22. Pérez-Alenza D, Melián C. Hyperadrenocorticism in dogs. In: Ettinger SJ, Feldman EC, Côté E, eds. Textbook of Veterinary Internal Medicine. 8th ed. St. Louis, MO: Elsevier; 2017:1795-1811.
23. Singh AK, Jiang Y, White T, Spassova D. Validation of nonradioactive chemiluminescent immunoassay methods for the analysis of thyroxine and cortisol in blood samples obtained from dogs, cats, and horses. *J Vet Diagn Invest.* 1997;9:261-268.
24. Scott-Moncrieff JC, Koshko MA, Brown JA, Hill K, Refsal KR. Validation of a chemiluminescent enzyme immunometric assay for plasma adrenocorticotropic hormone in the dog. *Vet Clin Pathol.* 2003;32:180-187.
25. Boretti FA, Holzthuem J, Reusch CE, et al. Lack of association between clinical sings and laboratory parameters in dogs with hyperadrenocorticism before and during trilostane treatment. *Schweiz Arch Tierheilkd* 2016;158:631-638.
26. Wehner A, Glöckner S, Weiss B, et al. Association between ACTH stimulation test results and clinical signs in dogs with hyperadrenocorticism treated with trilostane. *Vet J. 2021;276*:105740.
27. Arenas Bermejo C, Pérez Alenza D, García San José P, et al. Laboratory assessment of trilostane treatment in dogs with pituitary dependent hyperadrenocorticism. *J Vet Intern Med.* 2020;34:1413-1422.
28. Wenger M, Sieber-Ruckstuhl NS, Müller C, et al. Effect of trilostane on serum concentrations of aldosterone, cortisol and potassium in dogs with pituitary-dependent hyperadrenocorticism *Am J Vet Res* 2004;65:1245-12
29. King JB, Morton JM. Incidence and risk factors for hypoadrenocorticism in dogs treated with trilostane. *Vet J.* 2017;230:24–29.
30. Appleman E, Schrage A, Lamb KE, et al. Evaluation of iatrogenic hypocortisolemia following trilostane therapy in 48 dogs with pituitary-dependent hyperadrenocorticism. *J Am Anim Hosp Assoc.* 2021;*57*:217-224.
31. Lamoureux A, Cadoré JL, Hugonnard M, et al. (2023). Iatrogenic symptomatic hypoadrenocorticism after treatment with trilostane for hyperadrenocorticism in dogs: eight cases (2008-2019). *J Small Anim Pract.* 2023;64:409-414.
32. Macfarlane L, Parkin T, Ramsey I. Pre-trilostane and three-hour post trilostane cortisol to monitor trilostane therapy in dogs. *Vet Rec.* 2016;179:597-601.
33. Soulsby SN, Holland M, Hudson JA, et al. Ultrasonographic evaluation of adrenal gland size compared to body weight in normal dogs. *Vet Radiol Ultrasound*. 2015:56:317-326.
34. -2627. doi:10.1111/jvim.162

Chapter 8

Dopamine and somatostatin receptors and filamin A expression in normal pituitaries and corticotroph adenomas in dogs

Stefania Golinelli, Sara Galac, Björn Meij, Hans Kooistra, Federico Fracassi, Frank van Steenbeek, Karin Sanders

Winner as the Best Poster of the European Society of Veterinary Endocrinology, ECVIM-CA congress, Goteborg 2022

Dipartimento di Scienze Mediche Veterinarie,

Scuola di Agraria e Medicina Veterinaria,

Bologna

Introduction

Dopamine agonists and somatostatin analogs have been previously investigated as possible pituitary targeting drugs both in human and canine pituitary-dependent hypercortisolism (PDH). The expression of dopamine and somatostatin receptors subtypes, targets of dopamine agonists and somatostatin analogs, in canine corticotroph adenoma still needs to be clarified.[1,2] In humans, the actin binding protein filamin A (FLNA) is required for somatostatin receptor 2 (SSTR2) and dopamine receptor 2 (DRD2) expression and signaling in pituitary tumors, playing a role in tumor responsiveness to somatostatin receptors ligands and dopaminergic drugs.[3,4]

Objective

The purpose of this study was to evaluate the mRNA expression of the different dopamine and somatostatin receptor subtypes and FLNA in canine normal adenohypophysis (NAs) and in canine corticotroph adenoma (CAs). [1,2]

Methodology

Tissues from nine NAs and 32 CAs were included in the study. The CAs were collected from dogs with a diagnosis of PDH that underwent transsphenoidal hypophysectomy between 2015 and 2021. The NAs were collected from healthy dogs that were euthanized for reasons unrelated to this study. The gene expression levels of dopamine and somatostatin receptor subtypes and FLNA were evaluated with RNA sequencing. The expressions of dopamine and somatostatin receptors and FLNA were compared between NAs and Cas with the Mann-Whitney U test. The correlation between the mRNA expression of the dopamine receptors and somatostatin receptors and between the dopamine and somatostatin receptor and the FLNA were evaluated through the Spearman's rank correlation coefficient.

Results

mRNA of the DRD2 and the SSTR2 was detected in all NAs and in 31 out of 32 CAs (97%). None of the other annotated dopamine and somatostatin receptor subtypes were found to be expressed in either NAs or CAs. The NAs showed significantly higher expression of DRD2 ($P=8.3x10^{-5}$) (Figure1) and SSTR2 ($P=1.5x10^{-3}$) (Figure 2) in comparison with the CAs. FLNA was expressed in all samples and showed no significant difference in expression levels between NAs and CAs (P=0.64) (Figure 3). The DRD2 mRNA expression was significantly positively correlated with SSTR2 mRNA expression ($r=0.76$, $P=9.4x10^{-9}$) (Figure 4).

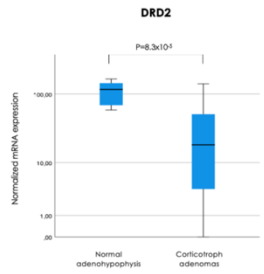

Figure 1: Box and whisker plots of DRD2 expression in normal anterior pituitaries (9) and corticotroph adenomas (n=32). The lower and upper boundaries of the box represent the first and third quartiles of the data, respectively, with the line within the box representing the median. The whiskers represent the 5th to 95th percentile.

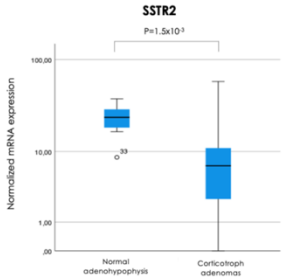

Figure 2: Box and whisker plots of SSTR2 expression in normal anterior pituitaries (9) and corticotroph adenomas (n=32). The lower and upper boundaries of the box represent the first and third quartiles of the data, respectively, with the line within the box representing the median. The whiskers represent the 5th to 95th percentile.

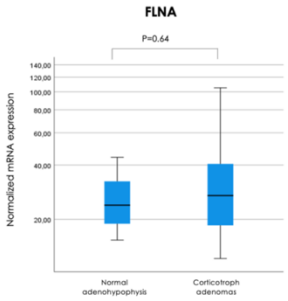

Figure 3: Box and whisker plots of FLNA expression in normal anterior pituitaries (9) and corticotroph adenomas (n=32). The lower and upper boundaries of the box represent the first and third quartiles of the data, respectively, with the line within the box representing the median. The whiskers represent the 5th to 95th percentile.

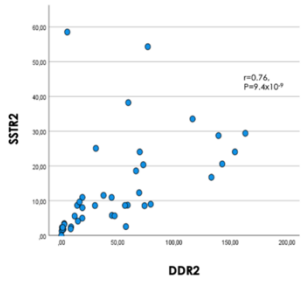

Figure 4: Correlation between DRD2 and SSTR2 expression

Conclusions

This study shows that DRD2 and SSTR2 were expressed in almost all CAs, albeit at lower levels than in NAs. FLNA, an acting-binding protein that is required for SSTR2 and DRD2 expression and signaling, was expressed in all CAs, indicating that these signaling pathways are potentially activated. Interestingly, DRD2 and SSTR2 expression levels were strongly correlated, highlighting the possibility of combined targeting of these receptor subtypes.

References

1. de Bruin C, Pereira AM, Feelders RA, et al. Coexpression of dopamine and somatostatin receptor subtypes in corticotroph adenomas. *J Clin Endocrinol Metab*. 2009;94:1118-1124.
2. Sato A, Hara Y. Protein expression of somatostatin receptor 2, somatostatin receptor 5 and dopamine D2 receptor in normal pituitary gland and ACTH-secreting pituitary adenoma in dogs. *Res Vet Sci.* 2018;119:61-66.
3. Mangili F, Treppiedi D, Catalano R. A novel mechanism regulating dopamine receptor type 2 signal transduction in pituitary tumoral cells: The role of cAMP/PKA-induced filamin A phosphorylation. *Front Endocrinol.* 2021;11:611752.
4. Treppiedi D, Di Muro G, Mangili F. Filamin A is required for somatostatin receptor type 5 expression and pasireotide-mediated signaling in pituitary corticotroph tumor cells. *Mol Cell Endocrinol.* 2021;524: 111159.

Chapter 9

Addition of cabergoline to trilostane treatment for dogs with pituitary-dependent hypercortisolism

Stefania Golinelli, Federico Fracassi, Álan Gomes Pöppl, Viviani De Marco, Diego Daniel Miceli, Kyoung won Seo, Edward C. Feldman

Winner as the Clinical Research Oral Presentation of the European Society of Veterinary Endocrinology, ECVIM-CA congress, Barcelona 2023

Dipartimento di Scienze Mediche Veterinarie,

Scuola di Agraria e Medicina Veterinaria,

Bologna

Introduction

Trilostane (T) is usually effective in controlling the hypercortisolemic state in canine pituitary-dependent hypercortisolism (PDH), however, its effect on pituitary tumor (PT) function and growth has not been reported. Cabergoline (C), a dopamine agonist, is a potential "pituitary-targeting" drug.[1]

Objective

This study aimed to evaluate the addition of cabergoline to trilostane in controlling PDH's clinical signs and/or blocking growth or even reducing the size of PTs.

Methodology

This prospective, controlled, multicenter study included 25 dogs with PDH (PT height [PTh] ≤12 mm). Thirteen dogs (TC group; TCg) were treated with T [median 0.5 mg/Kg (minimum 0.3-maximum 3.2)] and C (23 mcg/Kg q48h) (TC group, TCg) and 12 dogs with only T (T group, Tg) for at least 6 months. Each dog underwent a pituitary CT scan at the beginning (T0) and the end of the study (T180-T365); pituitary/brain ratio (PBr) was calculated from each scan. Each dog was monitored at T30 (days), T60, T120, T180, and T365 with a clinical evaluation (standardized questionnaire, higher scores were associated with worst PDH clinical control), urine specific gravity, cortisol (prepill or ACTH stimulation test) and endogenous ACTH (eACTH) measurement.

Results

Results of the questionnaire, USG, eACTH, and PBr were not significantly different between TCg and Tg at any time point. At T0 PTh was significantly higher (P=0.03) in the TCg versus the Tg. Questionnaire scores were significantly higher (P=0.01) at T30 versus T365 in the Tg. In the Tg the PBr were significantly higher (P=0.04) at T365 versus T0 (Figure 1). In the TCg, PTh was smaller in 4/12 [1.2 mm (0.7-4.7)]; PTh did not show any change in 2/12; PTh increased in 6/12 dogs [1.7 mm (1-4.2)]; and one dog died before the end of the study. In the Tg, PTh was smaller in 5/12 [0.17 mm (0.03-0.3)], was not visualized at either T0 or T365 in one, and it increased in 6/12 dogs [2 mm (1-5.7)]. In TCg the PBr reduced in 4/12 dogs [0.07 (0.01-0.13)] and increased in 8/12 dogs [0.08 (0.06-0.43)]. In the Tg, the PBr reduced in 3/12 [0.02 (0.01-0.03)], did not show any change in 3/12, and increased in 6/12 dogs [0.15 (0.06-0.35)].

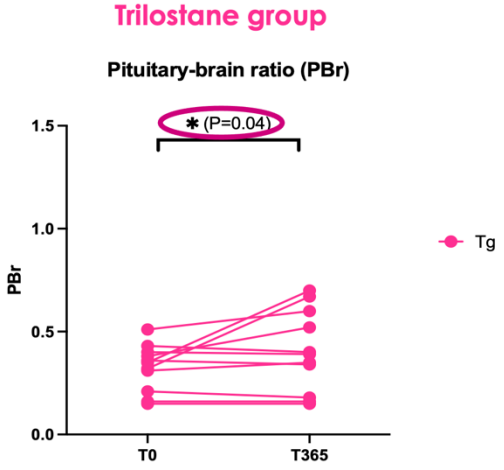

Figure 1: Pituitary-brain ratio at diagnosis and after 1 year in the Tg.

Discussion

The combination of trilostane and cabergoline treatment does not improve the control of PDH's clinical signs in comparison with trilostane treatment alone. However, cabergoline, potentially, plays a role in controlling the PT growth in PDH dogs.

References

1. Castillo VA, Gomez NV, Lalia JC, et al. Cushing's disease in dogs: cabergoline treatment. *Res Vet Sci* 2008;85:26-34.

Chapter 10

Summarizing discussion and conclusions

The present thesis aims to evaluate different clinical, clinicopathological, diagnostic, and therapeutic aspects of canine spontaneous hypercortisolism (HC). Hypercortisolism, also known as Cushing's syndrome, is defined by the physical and biochemical consequences resulting from prolonged exposure to elevated circulating levels of glucocorticoids.[1]

Typical clinical signs of HC are polyuria and polydipsia (PU/PD), increased appetite (polyphagia), abdominal enlargement, hair loss (alopecia), excessive panting, and muscle atrophy.[1]

Some dogs instead of showing muscle weakness, experience nonpainful severe muscle stiffness (SMS), resulting in a bilateral extremely stiff and stilted gait. Even in a prone position, affected dogs display severe and persistent extensor rigidity. Clinically, this combination of SMS in dogs with HC is referred to as "Cushing's myotonia" and has been described in less than 20 patients.[2-7] In **Chapter 3**, the findings of a retrospective study are presented, involving dogs concurrently affected by HC and SMS. The study involved collaboration with 14 colleagues from 10 institutions located in geographically diverse areas, resulting in the inclusion of only 37 dogs over 37 years. Considering the incidence of hypercortisolism at 1–2 cases per 1000 dogs per year, these results underscore the uncommon nature of this dual condition. The combination of HC and SMS possibly affects only dogs with pituitary-dependent hypercortisolism (PDH). The onset of SMS can manifest either before or after the diagnosis, and also after the treatment, of HC. Beyond the presence of SMS, the clinical presentation and survival duration of these dogs appear indistinguishable from those of dogs with HC in a broader context.[8-11] However, it is noteworthy that while muscle weakness typically resolves with HC treatment, the same resolution does not occur for SMS despite many treatments used.

In conclusion, the study presented in **Chapter 3** shed light on certain aspects of the coexistence of SMS and hypercortisolism HC. However, numerous uncertainties persist and await further resolution.

Cortisol influences calcium metabolism through various mechanisms. Dogs with HC exhibit some consequences of this condition such as calcium-containing urolithiasis. Additional abnormalities associated with disrupted calcium homeostasis in HC include hyperphosphatemia, elevated serum parathyroid hormone (PTH) levels, decreased urinary phosphate excretion, and heightened urinary calcium excretion.[12-16] The exact mechanism underlying these alterations is not yet fully comprehended**.** In **Chapter 4** the evaluation of the main regulators of calcium and phosphate homeostasis confirmed higher mean serum phosphate concentrations, median urinary fractional excretion of calcium (FECa), and median serum PTH concentrations in dogs with naturally occurring hypercortisolism (NOHC) at the time of diagnosis in comparison with healthy dogs. However, total calcium, ionized calcium, calcitriol, and urinary phosphate excretion did not differ between the above mentioned groups. The study introduced the calcitriol to 25-(OH)D ratio as a measure of vitamin D hydroxylation efficiency, suggesting increased efficiency in NOHC dogs. Unexpectedly, plasma fibroblast growth factor-23 (FGF-23) concentrations were lower in NOHC dogs, contrasting typical associations with hyperphosphatemia.[17,18] The clinical significance of reduced FGF-23 in hypercortisolemic dogs remains unclear, and the study speculates on its potential role in hyperphosphatemia and hypophosphaturia. However, uncertainties persist regarding the impact on phosphate metabolism, and factors like tissue expression of the Klotho:FGF receptor complex were not assessed. Overall, while the findings shed light on biochemical alterations in NOHC dogs, comprehensive understanding and clinical implications require further investigation.

The results of a cross-sectional survey study, assessing current testing protocols used by Western European primary care veterinarians (WEPCVs) for HC screening and differentiation are reported in **Chapter 5.** The research encompassed 2178 responses from nine European countries. In cases suspected of HC, 98.7% of respondents opted for endocrine testing, with 1.2% relying on a treatment trial. Among those conducting endocrine testing, 59.9% screened dogs for HC even in

the absence of consistent clinical signs but with clinicopathological abnormalities. Out of 2150 respondents performing endocrine testing, 66.6% consistently employed the same initial screening tests, with the ACTH stimulation test (34.8%), LDDST (30.4%), or a combination of tests (25.2%) being the most common. In cases without financial constraints, 66% of respondents always attempted differentiation, utilizing abdominal ultrasonography (81%) and LDDST (46.1%) most frequently. Overall, 69.8% of respondents referred suspected or diagnosed HC cases to internal medicine or dermatology specialists in ≤20% of instances over the previous five years. Testing protocols exhibited variation among Western WEPCVs. Notably, almost 60% of respondents potentially screened for HC in dogs lacking consistent clinical signs, raising concerns about potential overdiagnosis. A portion of WEPCVs refrained from attempting differentiation, likely impacting management strategies and prognosis. Referral to specialists was infrequent, indicating that HC is predominantly managed in first-opinion practices. These findings underscore the need for further education among WEPCVs.

Trilostane has been the medical treatment of choice for PDH for the past two decades, acting as a competitive inhibitor of the 3β-hydroxysteroid dehydrogenase/isomerase system involved in cortisol, aldosterone, and androstenedione synthesis. Proper dosing and frequency effectively manage clinical signs and associated abnormalities in HC.[5] The adrenocorticotropic hormone stimulation test (ACTHst) has traditionally monitored trilostane treatment, but concerns about its reliability have emerged over time. Consequently, alternative methods for monitoring trilostane treatment have been explored in the last decade.[19-25] Among these, prepill serum cortisol concentration, urine specific gravity (USG), and haptoglobin (Hp) have shown promising results, despite certain limitations.[19-21] Studies emphasize the significance of clinical evaluation to distinguish well-controlled from undercontrolled trilostane-treated dogs. However, it is acknowledged that assessments by inexperienced owners or clinicians may sometimes be unreliable. **Chapter 6** shows the results of a study aimed to evaluate and compare the ability of 12 possible methods for monitoring trilostane treatment to identify the clinical control correctly and objectively in dogs classified as well-controlled, undercontrolled, and unwell. Ninety-four reassessments of 44 dogs were analyzed, excluding 5 reassessments of unwell dogs. Haptoglobin demonstrated a significant association with the clinical score ($P < .001$). In the receiver operating characteristic analysis, a Hp cutoff of 151 mg/dL accurately identified 90.0% of well-controlled dogs (specificity) and 65.6% of undercontrolled dogs (sensitivity). Alanine aminotransferase (ALT) ($P = .01$) and γ-glutamyl transferase (γGT) ($P = .009$) were notably elevated in undercontrolled dogs. Cutoff values of ALT and γGT greater than or equal to 86 U/L and 5.8 U/L, respectively, were significantly associated with inadequate control of hypercortisolism by trilostane.
Of all the 12 variables, Hp, and to a lesser degree ALT and γGT, could be considered additional tools to identify well-controlled and undercontrolled trilostane-treated dogs. However, none of the tests can serve as a replacement for clinical assessment.

The main objectives in treating HC include achieving normocortisolism, resolving clinical signs, mitigating complications, improving quality of life, and addressing the root cause of excessive cortisol production.[26] Published studies show that trilostane is effective in achieving these goals, although complete elimination of the underlying source is not possible. However, control of clinical signs varies, with good control reported in 50% to 100% of treated dogs within a few weeks and over 75% showing partial to complete control after several months.[27-35] About 10-15% of dogs treated with trilostane may not exhibit improvement.[1] **Chapter 7** reports results of retrospective study with the goal to compare findings between dogs with favorable and poor responses to trilostane treatment.

Twenty-four dogs were categorized as good responder dogs (GRDs), while 10 were classified as partially responsive dogs (PRDs). In GRDs, several variables were significantly lower at diagnosis compared to PRDs, including ALT (P=.004), γGT (P=.039), phosphate (P=.039), endogenous ACTH (P=.027), pre-ACTH cortisol (P=.004), and 8-hour post-dexamethasone cortisol (T8) (P=.019). Additionally, creatinine and the starting dose of trilostane were significantly lower and higher in GRDs (P=.006; P=.021), respectively. The proportion of GRDs without bilateral adrenomegaly was significantly higher than PRDs (P=0.003). Among clinical signs, alopecia was significantly more observed in PRDs at diagnosis (P=0.015). At 6 months, several variables in GRDs were significantly lower compared to PRDs, including ALT (P<.001), γGT (P=0.005), ALP (P=0.005), cholesterol (P<.001), phosphate (P=0.022), pre-ACTH cortisol (P<.001), and post-ACTH cortisol (P<.001). Creatinine was significantly higher in GRDs (P=0.003). The study suggests that routinely performed clinical-pathological variables associated with certain clinical and ultrasonographic findings may aid in identifying PRDs.

In summary, dogs presenting with more severe clinical signs and unfavorable clinicopathological findings are inclined to show resistance to trilostane treatment. Elevated levels of ALT, gGT, phosphorus, pre-ACTH cortisol, T8 cortisol, endogenous ACTH, and the initial trilostane dose, along with lower creatinine concentrations, indicate a higher likelihood of an inadequate response to therapy. These findings suggest the need for prospective studies to validate and delve deeper into the gathered data.

Lastly **Chapters 8** and **9** focus on the results of two abstracts investigating possible alternative pituitary targeting drugs for canine PDH. In particular, in **Chapter 8** the mRNA expression of all the different dopamine (DRD2) and somatostatin (SSTR2) receptor subtypes and the actin-binding protein filamin A (FLNA) in canine normal adenohypophysis (NAs) and corticotroph adenomas (CAs) are evaluated. The mRNA of DRD2 and SSTR2 was found in the majority of CAs, though at lower levels than in normal adenohypophysis (NAs). The FLNA, a key protein for DRD2 and SSTR2 expression and signaling, was expressed in all CAs, suggesting potential pathway activation. NAs exhibited significantly higher expression of DRD2 and SSTR2. Interestingly, DRD2 and SSTR2 expression levels were strongly correlated, indicating the possibility of a combined therapeutic approach targeting both receptor subtypes.

Chapter 9 shows the preliminary results of a prospective, controlled, multicenter study that evaluated the addition of cabergoline to trilostane in resolving PDH's clinical signs and/or blocking growth or even reducing the size of pituitary tumors. The study involved 25 dogs with PDH, and two treatment groups were compared: one receiving both trilostane and cabergoline (TC group), and the other receiving only trilostane (T group) for at least 6 months. The study assessed various parameters, including clinical evaluations, urine specific gravity, endogenous ACTH levels, and pituitary/brain ratio (PBr) from CT scans, at different time points.

Results indicated no significant differences in clinical signs control between the TC group and the T group. However, the TC group showed potentially beneficial effects on controlling pituitary growth (measured by pituitary height) compared to the T group. Overall, while the combined treatment did not improve clinical signs control, cabergoline might play a role in managing the growth of the pituitary gland in dogs with PDH.

Conclusions

- Severe muscle stiffness (SMS) is a rare condition that affects dogs with PDH. To date, no efficacy treatments have been found, despite the median survival time of dogs with PDH and SMS being similar to that of dogs with only PDH.

- Hyperphosphatemia, urine calcium excretion, and increased PTH play a role in adrenal secondary hyperparathyroidism.
- Around 60% of WEPCVs screen dogs for HC without consistent clinical signs and a proportion of them never attempt differentiation. The results underscore potential concerns about overdiagnosis and highlight a need for further education among WEPCVs in managing HC.
- The clinical picture is essential and cannot be replaced by laboratory variables when monitoring trilostane treatment in dogs with HC. Haptoglobin, along with ALT and γGT, may be considered supplementary tools. However, none of these variables has the capacity to identify overdosed dogs.
- Dogs presenting a more severe clinical picture and exhibiting more pronounced clinicopathological abnormalities are inclined to show resistance to trilostane treatment. Elevated concentrations of ALT, γGT, phosphorus, pre-ACTH cortisol, T8 cortisol, endogenous ACTH (eACTH), and the initial trilostane dose, alongside lower creatinine concentrations, enhance the likelihood of an inadequate response to therapy.
- DRD2 and SSTR2 are detected in most CAs. FLNA is present in all CAs, suggesting potential activation of these signaling pathways.
- The combination of trilostane and cabergoline treatment does not improve the control of PDH's clinical signs but cabergoline, potentially, plays a role in controlling the PT growth in PDH dogs.

References

1. Behrend EN. Canine hyperadrenocorticism. In: Feldman EC, Nelson RW, Reusch CE, et al., eds. Canine and Feline Endocrinology. 4th ed. St. Louis, MO: Elsevier Saunders; 2015:377-451.
2. Griffiths IR, Duncan ID. Myotonia in the dog: a report of four cases. *Vet Rec.* 1973;93:184-188.
3. Duncan ID, Griffiths IR, Nash AS. Myotonia in canine Cushing's disease. *Vet Rec.* 1977;100:30-31.
4. Greene CE, Lorenz MD, Munnell JF, et al. Myopathy associated with hyperadrenocorticism in the dog. *J Am Vet Med Assoc.* 1979;174:1310-1315
5. Swinney GR, Foster SF, Church DB, et al. Myotonia associated with hyperadrenocorticism in two dogs. *Aust Vet J.* 1998;76:722-724
6. Nagata N, Yuki M. Long-term outcome of myotonia associated with hyperadrenocorticism in 2 dogs. *Can Vet J.* 2015;56:931-933.
7. Nam S, Kang BT, Song KH, Seo KW. Long-term follow up of refractory myotonia associated with hyperadrenocorticism in a Maltese dog. *J Vet Clin.* 2020;37:273-277.
8. Barker E, Campbell S, Tebb A, et al. A comparison of the survival times of dogs treated with mitotane or trilostane for pituitary-dependent hyperadrenocorticism. *J Vet Intern Med.* 2005;19:810-815.
9. Clemente M, De Andrés PJ, Arenas C, et al. Comparison of nonselective adrenocorticolysis with mitotane or trilostane for the treatment of dogs with pituitary-dependent hyperadrenocorticism. *Vet Rec.* 2007;161:805-809.
10. Fracassi F, Corradini S, Floriano D, et al. Prognostic factors for survival in dogs with pituitary-dependent hypercortisolism treated with trilostane. *Vet Rec*. 2014;176:49.
11. García San José P, Arenas C, Alonso-Miguel D, et al. Survival of dogs with pituitary-dependent hyperadrenocorticism treated twice daily with low doses of trilostane. *Vet Rec.* 2022;191:e1630.
12. Hess RS, Kass PH, Ward CR. Association between hyperadrenocorticism and development of calcium-containing uroliths in dogs with urolithiasis. *J Am Vet Med Assoc.* 1998;212:1889-1891.

13. Ramsey IK, Tebb A, Harris E, Evans H, Herrtage ME. Hyperparathyroidism in dogs with hyperadrenocorticism. *J Small Anim Pract.* 2005;46:531-536.
14. Tebb AJ, Arteaga A, Evans H, Ramsey IK. Canine hyperadrenocorticism effects of trilostane on parathyroid hormone, calcium and phosphate concentrations. *J Small Anim Pract*. 2005;46: 537-542.
15. Fracassi F, Malerba E, Furlanello T, Caldin M. Urinary excretion of calcium and phosphate in dogs with pituitary-dependent hypercortisolism: case control study in 499 dogs. *Vet Rec*. 2015;117:625.
16. Mooney CT, Shiel RE, Sekiya M, et al. A preliminary study of the effect of hyperadrenocorticism on calcium and phosphate concentrations, parathyroid hormone and markers of bone turnover in dogs. *Front Vet Sci.* 2020;7:1-9.
17. de Brito Galvao JF, Nagode LA, Schenck PA, Chew DJ. Calcitriol, calcidiol, parathyroid hormone, and fibroblast growth factor-23 interactions in chronic kidney disease. *J Vet Emerg Crit Care.* 2013;23:134-162.
18. Takashi Y, Fukumoto S. Phosphate-sensing and regulatory mechanism of FGF23 production. *J Endocrinol Invest*. 2020;43:877-883.
19. Macfarlane L, Parkin T, Ramsey I. Pre-trilostane and three-hour post trilostane cortisol to monitor trilostane therapy in dogs. *Vet Rec.* 2016;179:597-601.
20. Arenas Bermejo C, Pérez Alenza D, García San José P, et al. Laboratory assessment of trilostane treatment in dogs with pituitarydependent hyperadrenocorticism. J Vet Intern Med. 2020;34:1413-1422.
21. McGrotty YL, Arteaga A, Knottenbelt CM, et al. Haptoglobin concentrations in dogs undergoing trilostane treatment for hyperadrenocorticism. *Vet Clin Pathol.* 2005;34:255-258.
22. Galac S, Buijtels JJ, Kooistra HS. Urinary corticoid:creatinine ratios in dogs with pituitary-dependent hyperadrenocorticism during trilostane treatment. *J Vet Intern Med.* 2009;23:1214-1219.
23. Arteaga A, Dhand NK, McCann T, et al. Monitoring the response of canine hyperadrenocorticism to trilostane treatment by assessment of acute phase protein concentrations. *J Small Anim Pract.* 2010;51:204-207.
24. Cook AK, Bond KG. Evaluation of the use of baseline cortisol concentration as a monitoring tool for dogs receiving trilostane as a treatment for hyperadrenocorticism. *J Am Vet Med Assoc*. 2010;237: 801-805.
25. Burkhardt WA, Boretti FS, Reusch CE, Sieber-Ruckstuhl NS. Evaluation of baseline cortisol, endogenous ACTH, and cortisol/ACTH ratio to monitor trilostane treatment in dogs with pituitary-dependent hyperadrenocorticism. *J Vet Intern Med.* 2013;27:919-923.
26. Sanders K, Kooistra HS, Galac S. Treating canine Cushing's syndrome: current options and future prospects. *Vet J.* 2018;241:42-51.
27. Ruckstuhl NS, Nett CS, Reusch CE. Results of clinical examinations, laboratory tests, and ultrasonography in dogs with pituitary-dependent hyperadrenocorticism treated with trilostane. *Am J Vet Res*. 2002;63:506–512.
28. Galac S, Buijtels JJCWM, Mol JA, Kooistra HA. Effects of trilostane treatment on the pituitary-adrenocortical and renin-aldosterone axis in dogs with pituitary-dependent hypercortisolism. *Vet J.* 2008, doi:10.1016/j.tvjl.208.10.007
29. Braddock JA, Church DB, Robertson ID, Watson AD. Trilostane treatment in dogs with pituitary-dependent hyperadrenocorticism. *Aust Vet J.* 2003;81:600–607.
30. Augusto M, Burden A, Neiger R, Ramsey I. A comparison of once and twice daily administration of trilostane to dogs with hyperadrenocorticism. *Tierarztl Prax K H.* 2012;40:415–424.
31. Arenas C, Melian C, Perez-Alenza MD. Evaluation of 2 trilostane protocols for the treatment of canine pituitary-dependent hyperadrenocorticism: Twice daily versus once daily. *J Vet Intern Med.* 2013;27:1478-1485.

32. Cho KD, Kang JH, Chang D, Na KJ, Yang MP. Efficacy of low-and high-dose trilostane treatment in dogs (< 5 kg) with pituitary-dependent hyperadrenocorticism. *J Vet Intern Med.* 2013;27:91–98.
33. Alenza DP, Arenas C, Lopez ML, Melian C. Long-term efficacy of trilostane administered twice daily in dogs with pituitary-dependent hyperadrenocorticism. *J Am Anim Hosp Assoc.* 2006;42:269–276.
34. Vaughan MA, Feldman EC, Hoar BR, Nelson RW. Evaluation of twice-daily, low-dose trilostane treatment administered orally in dogs with naturally occurring hyperadrenocorticism. *J Am Vet Med Assoc.* 2008;238:1441-1451.
35. Feldman EC. Evaluation of twice-daily lower-dose trilostane treatment administered orally in dogs with naturally occurring hyperadrenocorticism. *J Am Vet Med Assoc.* 2011;238:1441–1451.