Interpretation of Laboratory Tests for Canine Cushing's Syndrome

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Hypercortisolism (HC) is a common disease in dogs. This article will review the laboratory tests that are available for diagnosis of HC and laboratory tests for differentiating between causes of HC. An emphasis will be made on the clinical process that leads to the decision to perform those tests and common misconceptions and issues that arise when performing them. To choose between the adrenocorticotropic hormone (ACTH)–stimulation test and the low-dose dexamethasone suppression test (LDDST), the advantages and disadvantages of both tests should be considered, as well as the clinical presentation. If the index of suspicion of HC is high and other diseases have been appropriately ruled out, the specificity of the ACTH stimulation test is reasonably high with an expected high positive predictive value. Because of the low sensitivity, a negative result in the ACTH stimulation test should not be used to rule out the diagnosis of HC. The LDDST is more sensitive but also less specific and affected more by stress. A positive result on the urine cortisol:creatinine ratio does not help to differentiate HC from other diseases. A negative result on the urine cortisol:creatinine ratio indicates that the diagnosis of HC is very unlikely. The LDDST is useful in differentiating pituitary-dependent HC from an adrenal tumor in about two thirds of all dogs with HC. Differentiation of HC from diabetes mellitus, liver diseases, and hypothyroidism cannot be based solely on endocrine tests. Clinical signs, imaging studies, histopathology, and response to treatment should all be considered.

Keywords: dexamethasone, ACTH, cortisol, hyperadrenocorticism, hyperadrenocortisolism

Cushing's syndrome is an almost pathognomonic constellation of less-than-subtle clinical signs and is often diagnosed easily by measuring cortisol after stimulation or suppression of the pituitary-adrenal axis. The more subtle cases of Cushing's syndrome can be difficult to diagnose, and the interpretation of dynamic adrenal function tests is complicated when other diseases are present. Hypercortisolism (HC) is a clinical syndrome caused by the multisystemic effects of abnormally high circulating concentrations of glucocorticoids. In veterinary medicine, the term "hyperadrenocorticism" is used more commonly than "hypercortisolism," but because there are clinical syndromes associated with high circulating concentrations of adrenocortical hormones other than cortisol (e.g., aldosterone, estradiols, cortisol precursors, estradiol precursors), the term "hypercortisolism" more accurately reflects the clinical syndrome discussed here. Increased cortisol secretion is also a part of a normal stress response to injury or disease. It is not uncommon for clinicians to associate stress-related abnormalities with HC and to screen for HC when those abnormalities are present, leading to misdiagnosis. This article will review the laboratory tests that are available for diagnosis of HC and laboratory tests for differentiating between causes of HC. An emphasis will be made on common misconceptions and issues that arise when performing those tests, regardless of the reason to perform them or their outcome. Other aspects of Cushing's syndrome, such as pathophysiology, diagnostic imaging, and treatment, will be discussed when pertinent to the interpretation of laboratory tests.

Cortisol has diverse effects on virtually all body tissues and its effects vary with the concentration. The clinical signs of HC are caused by the combined glucogenic, immune-suppressive, antiinflammatory, protein-catabolic, and lipolytic effects of cortisol.[1] One important effect of cortisol is to provide a negative feedback signal, inhibiting pituitary adrenocorticotropic hormone (ACTH) release.[2,3] Physiological concentrations of cortisol are essential for maintenance of normal body functions, and appropriate increases in cortisol concentrations are part of a normal response to stress of injury or disease. In chronic stress, the effects of cortisol are essentially the same as in HC but are masked or overridden by the effects of the primary disease condition. Depending on the primary disease, some overlap in clinical signs might exist

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Table 1. Clinical Signs in Dogs with HC (Modified from[1,4])

	Number of Cases	
	N > 2000[4]	N > 450[1]
Clinical Signs	%	%
Polydipsia/Polyuria	80-85	80-91
Polyphagia	90	46-57
Pendulous abdomen	> 80	67-73
Muscle weakness	75-85	14-57
Alopecia	33	60-74
Testicular atrophy/Anestrus	N/R	29-54
Muscle atrophy	N/R	35
Comedones/Hyperpigmentation	N/R	23-34
Panting	N/R	30
Calcinosis cutis	N/R	8-15
Facial nerve paralysis	N/R	7

Abbreviation: N/R, Not reported.

(e.g., polyuria [Pu] and polydipsia [Pd] in renal failure). Importantly, it is not uncommon to observe the full range of biochemical effects of cortisol, as well as abnormalities on functional tests of the pituitary-adrenal axis, when HC is not present and another disease is the cause of the chronic hypercortisolemia (e.g., in diabetes mellitus [DM]). Therefore, it is the combination of the biological effects of excessive cortisol with the absence of any other disease condition that gives the typical presentation of Cushing's syndrome. Before considering functional tests for the diagnosis of HC, it is important to recognize the clinical signs and biochemical abnormalities that are associated with the syndrome, as well as clinical signs and biochemical abnormalities that are not.

Patient Selection

Clinical Signs

Clinical signs of Cushing's syndrome are presented in Table 1 in order of frequency.[1,4] A list of clinical signs that should sway the clinician away from screening for Cushing's syndrome are presented in Table 2.[4] Most dogs with HAC are over 6 years of age.[1] Dogs with HC might have one or several clinical signs, each of which might vary in severity.[1] In the vast majority of dogs, the course of HC is insidious and slowly progressive. Rarely, clinical signs are intermittent.

Most dogs with Cushing's syndrome are presented with polyuria (Pu) and polydipsia (Pd). Pu/Pd can be easily overlooked or misinterpreted by owners, especially in early stages, and it is not uncommon for dogs with Cushing's syndrome to be presented for urinary incontinence or nocturia and enuresis. Polyphagia is the second most common clinical sign, and although it might not be present in all dogs with Cushing's syndrome, it is important to remember that decreased appetite is not a clinical sign of Cushing's syndrome. Decreased appetite can be seen in longstanding cases as a result of macroadenomas in the pituitary gland that have grown big enough to compress the hypothalamus. This typically happens years after classical signs of Cushing's syndrome have been detected.

Alopecia is a common presenting complaint and sometimes will concern owners more than Pu/Pd and polyphagia. The alopecia is typically truncal and bilaterally symmetric. Other, less common, dermatological signs include thin skin, failure to regrow hair in shaved areas, hyperpigmentation, comedones, calcinosis cutis, and seborrhea. Secondary pyoderma, calcinosis cutis, and seborrhea might cause pruritus, but this is uncommon.[4] It is a common mistake to screen dogs with allergic skin disease for HC. These dogs typically have chronic intermittent pruritus for years but no other signs consistent with HC. HC is suspected when treatment fails. It is important to remember that in a dog with chronic allergic skin disease, HC should, if anything, alleviate pruritis and not worsen it. Also, on treatment of HC, the allergic disease is likely to relapse.

Pendulous abdomen is a common presenting problem in dogs with HC. It is caused by a combination of weakened abdominal muscles, hepatomegaly, urine-filled bladder, and fat redistribution into the mesentery. This gives the impression of weight gain, but dogs with HC do not always gain much weight from the disease alone. Obesity is common in the general canine population and obesity is common in human patients with HC, but the relationship between obesity and HC in the dog has not been well established. Generalized muscle wasting is common and can cause lethargy in dogs with HC. Lethargy is also a common sign in dogs with pituitary macroadenomas. Obesity frequently and wrongly prompts clinicians to screen for HC, especially when owners complain about lethargy and decreased activity as well. Obesity, however, is far more common than HC, and the vast majority of obese dogs do not have Cushing's syndrome. Obese dogs are often less active than nonobese dogs. Screening dogs with obesity and decreased activity for HC is likely to result in a high rate of false-positive results because obese dogs have been shown to have exaggerated cortisol responses to exogenous ACTH.[5] Lethargy as a sole clinical sign will also sometimes prompt practitioners to screen for HC despite being an extremely unlikely clinical presentation. Other diseases should be considered first and the investigation should

Table 2. Clinical Signs That Should Sway the Clinician from Screening for Cushing's Syndrome (Modified from[4])

Decreased appetite	Vomiting	Diarrhea	Sneezing	Coughing
Icterus	Pruritis	Pain	Seizures	Bleeding

begin with a complete blood count, a full biochemistry profile, and a urinalysis.

Older dogs presented with seizures, blindness, and signs of HC are sometimes assumed to have pituitary macroadenomas. Seizures and blindness are uncommon in dogs with pituitary tumors, and their onset should prompt the clinician to look for other causes.[6] Clinical signs that are typically associated with pituitary macroadenomas include lethargy, inappetance, and mental dullness. Rarely, dogs with HC are presented with facial paralysis.[1]

Dogs with HC are rarely presented with critical illness and are rarely believed to be seriously ill by their owners.[4] Uncommon complications of hypercortisolism include pulmonary thromboembolism and hemorrhagic shock.[1] Even in those cases that are presented as emergencies, it is important to remember that the diagnosis and treatment of HC are not urgent and that no laboratory test can reliably distinguish HC from other causes of acute disease in those critical cases. False-positive results of diagnostic tests for HC are common in these cases, and it is best to stabilize the patient and search for other causes of the critical condition. Once resolved, if the index of suspicion of HC is high, a confirmatory test can be considered.

Clinicopathological Abnormalities

Hemogram High blood cortisol concentrations commonly cause a stress leukogram: lymphopenia, neutrophilia without a left shift, monocytosis, and eosinopenia. Among these changes, lymphopenia is most common and can be the only sign of a stress leukogram. Although commonly seen in dogs with Cushing's syndrome, a stress leukogram is not specific and can occur secondary to any other disease process. Mild erythrocytosis and thrombocytosis are also common in dogs with Cushing's syndrome. Although none of these abnormalities on the hemogram are specific for Cushing's syndrome, opposite findings should steer the clinician away from screening for Cushing's syndrome. In particular, anemia, thrombocytopenia, and neutropenia are likely associated with diseases that will cause false-positive results on tests for Cushing's syndrome.

Biochemistry Increased activity of serum alkaline phosphatase (ALP) is the most common biochemical finding in dogs with Cushing's syndrome and is present in about 90% of dogs. Marked elevations are common, but there is no correlation between the degree of elevation and the severity of the disease, response to treatment, or prognosis.[1] Because dogs have a steroid-induced ALP isoenzyme, it is a common misconception that the higher the ALP, the more likely the diagnosis of Cushing's syndrome, especially when other liver enzymes are normal. First, a normal ALP should not be used to exclude the diagnosis of Cushing's syndrome in a dog that otherwise has a consistent clinical presentation (about 10% of dogs with Cushing's syndrome have normal ALP activity). Second, an elevation in ALP activity is not specific for Cushing's syndrome even when other liver enzyme activities are normal. Any illness or injury causing stress can lead to an elevation in ALP activity if it causes an elevation in serum cortisol concentrations. ALP activity can also be increased by medications (e.g., phenobarbital) and aging.[1,7] In fact, elevated ALP activity is the most common abnormality on routine biochemical screening profiles and is considered the least liver-specific.[7] An increased ALP activity can be the only manifestation of hepatic nodular hyperplasia (HNH), a common finding in older dogs.[8] This benign condition is diagnosed in dogs as young as 6 years of age and is present in over 70% of dogs after the age of 14 years. Serum ALP activity in dogs with HNH is typically 2.5 times higher than the upper end of the reference interval, but 10- to 14-fold increases have been reported. It is a common mistake to suspect Cushing's syndrome in older dogs with increased ALP activity but without clinical signs of HC. If no clinical signs are present, the diagnosis of HNH is much more likely and no tests are needed. It is also worth noting that histologically, vacuolar changes are common in HNH. Vacuolar hepatopathy is also seen in Cushing's syndrome as a result of chronic elevation in cortisol concentrations, and, as with increased ALP activity, it is not specific for Cushing's syndrome and does not warrant screening tests for Cushing's syndrome. In a recent retrospective study on vacuolar hepatopathy in dogs, high ALP activities and vacuolar hepatopathy were commonly observed in a myriad of diseases, but only 55% of all dogs with high ALP had Cushing's syndrome or were exposed to exogenous steroids and only 12% of all dogs with a histopathological diagnosis of vacuolar hepatopathy had adrenal gland dysfunction.[9] These findings supported the authors' hypothesis that stress-induced hypercortisolemia associated with acute or chronic illness may contribute to the development of vacuolar hepatopathy and increased ALP activity in dogs.

Serum bile acid concentrations are a sensitive test for liver dysfunction. Because there is extensive overlap in the degree of bile acid increase between primary liver diseases and secondary causes of liver dysfunction, and because serum bile acids are commonly abnormal in dogs with Cushing's syndrome, measurement of bile acids is not helpful in differentiating Cushing's syndrome from primary liver disease.[9]

The exact frequency of increased serum GGT activity has not been reported in HC but it is common. Like ALP, GGT activities can be increased by exogenous glucocorticoid administration in dogs,[7] but the magnitude of increase in GGT activity in HC is typically less dramatic. Other common abnormalities that might be attributable to hepatopathy in dogs with HC include increased serum alanine aminotransferase activity and hyperlipidemia (in about 50%-80%) as well as low blood urea nitrogen (in 30%-50%).[1] The combination of these biochemical changes, together with increased ALP, GGT, and bile acids, as discussed before, is consistent with liver disease. Further discussion of differentiating liver disease from HC is given below.

Low serum phosphorus, mild hypernatremia, and mild hypokalemia are sometimes found in dogs with HC, but the clinical significance of these abnormalities is minimal. Hyperglycemia occurs in about 30% of dogs with HC, whereas

glucosuria and DM occur in up to 10%. Diagnosis of HC in diabetic dogs is discussed below.

Urinalysis Obtaining a sterile sample for urinalysis and a urine culture is essential in all suspected cases of HC. Urine-specific gravity is usually less than 1.020 and often it is less than 1.008.[4,10] Dogs with HC, however, can concentrate urine to some degree if they are deprived of water. Therefore, a urine sample taken early in the morning or after many hours in the hospital, with a specific gravity of 1.035, should not be used to rule out the possibility that the dog is Pu/Pd. In these cases it is recommended to quantify water intake over 48 hours at home. Significant proteinuria is found in the majority of dogs with HC, with a urine protein:creatinine ratio typically between 1.0 and 6.0 and sometimes higher. The urine protein:creatinine ratio typically decreases with treatment of HC but does not always normalize. Importantly, despite significant proteinuria, serum albumin is usually normal in dogs with HC. If hypoalbuminemia is present, other diagnoses should be considered. Urinary tract infections are found in about 50% of dogs with HC on presentation, but signs of lower urinary tract disease (pollakuria, stranguria, etc.) are typically absent and the urine sediment is usually inactive. Therefore, urine cultures should always be a part of the workup in a dog with suspected HC.

Test Selection: Diagnosis

The accuracy of a diagnostic test is determined by its sensitivity and specificity. Sensitivity and specificity are not useful, however, when attempting to predict the likelihood of a patient having the disease. For that, positive and negative predictive values (PPV and NPV, respectively) should be calculated (Table 3). The sensitivity of a test is defined as the proportion of positive test results in a population of patients that truly have the disease (the sum of true positives and false negatives). Therefore, the sensitivity can only be estimated in a population of patients that have the disease (based on confirmation by a gold standard test). This is obviously not helpful in practice. For the clinician, it is more important to know the proportion of true positives out of all the positive test results (the sum of true positives and false positives). This proportion is the PPV of the test and it depends on the accuracy of the test as well as on the prevalence of the disease in the population tested. Similarly, the specificity of a test is defined as the proportion of negative test results in a population of patients that do not have the disease (the sum of true negatives and false positives). Therefore, the specificity can only be estimated when the negative disease status of each and every patient is already confirmed by a gold standard test. Again, this is not helpful in practice. For the clinician, it is more important to know the proportion of true negatives out of all the negative test results (the sum of true negatives and false negatives). This proportion is the NPV of the test, and, like the PPV, it depends on the accuracy of the test as well as on prevalence of the disease in the population tested. Thus, for a test with a given sensitivity and specificity, the PPV increases and the NPV decreases when the prevalence increases. For endocrine diseases, the prevalence of the disease in the tested population is highly dependent on patient selection. If a test for confirming Cushing's syndrome is performed only on patients with the classic Cushingoid appearance, a positive result is more likely to be a true positive, and a negative result is less likely to be a true negative, regardless of the sensitivity and specificity of the test. Here we will discuss the sensitivity and specificity of diagnostic tests, but when interpreting results of such tests, appropriate patient selection, as discussed above, is imperative.

The first and most important screening test in the process of diagnosing HC (and perhaps any other disease) is the collection of signalment, history, and physical examination findings. Based on these, a suspicion of HC should be raised. A complete blood count, biochemistry panel, and a urinalysis with a urine culture should follow, and their results can increase or decrease the index of suspicion. Some findings in this process should prompt the clinician to consider other differential diagnoses first, as discussed above, even if a suspicion of HC is still present. If no other disease process is suspected, a confirmatory test should be performed. If the confirmatory test is positive, a differentiation between causes of HC is necessary.

Pathophysiology of the Hypothalamus-Pituitary-Adrenal Gland Axis in the Context of Endocrinological Tests

HC in the dog can be caused by either an ACTH-secreting tumor of the pituitary gland, a cortisol-secreting tumor of the adrenal cortex, or exogenous administration of glucocorticoid drugs. Other causes such as ectopic ACTH production

Table 3. Sensitivity, Specificity, and Predictive Values

	Disease Status (as determined by a gold standard test)		
	Positive	Negative	
Test result			
Positive	True positive	False positive	PPV = TP/(TP+FP)
Negative	False negative	True negative	NPV = TN/(TN+FN)
	Sensitivity = TP/(TP+FN)	Specificity = TN/(TN+FP)	

Abbreviations: PPV, Positive predictive value; TP, true positive; FP, false positive; NPV, negative predictive value; TN, true negative; FN, false negative.

and food-dependent hypercortisolemia have been described but are exceedingly rare.[11,12] In health, ACTH secretion is pulsatile and is positively controlled by corticotrophin-releasing hormone (CRH) and arginine vasopressin (or antidiuretic hormone) and negatively controlled by cortisol. Psychologic and physiologic stresses, as well as inflammatory cytokines, stimulate the hypothalamus to secrete CRH and arginine vasopressin. The cumulative effect of ACTH on the adrenal cortex over time determines the size of the latter and the overall capacity to synthesize and secrete cortisol. In the short term, stress causes a rapid increase in ACTH concentrations, which stimulate cortisol secretion within minutes. ACTH also stimulates the synthesis and secretion of androgens and aldosterone from the adrenal cortex, but this has little clinical significance. Basal concentrations of cortisol vary dramatically throughout the day (and therefore are not useful in diagnosis of HC). In health, cortisol inhibits ACTH release.

Pituitary-dependent HC (PDH) occurs in about 85% of dogs with naturally occurring HC. PDH is characterized by decreased responsiveness to the inhibitory effects of cortisol and overproduction of ACTH. Notably, both of these vary substantially with the degree of aberration of the tumor cells. The chronic increase in ACTH concentrations causes symmetric adrenocortical hyperplasia and leads to an overall increased capacity of the adrenal glands to respond to ACTH and secrete cortisol. Adrenal tumors (AT) occur in about 15% of dogs with HC. Secretion of cortisol from these tumors is spontaneous and independent of ACTH stimulation, although some residual responsiveness to ACTH persists to a varying degree. In AT, increased concentrations of cortisol suppress CRH and ACTH secretion and over time lead to atrophy of all nonneoplastic cells of the adrenal cortex, bilaterally. Rare cases of simultaneous occurrence of bilateral AT, PDH, and unilateral AT and PDH and bilateral AT have been described.[4]

Iatrogenic HC is characterized by chronically suppressed CRH and ACTH concentrations leading to bilateral cortical atrophy of the adrenal glands. Because of the bilateral atrophy, the overall capacity to respond to ACTH is decreased or abolished. The development of iatrogenic HC depends on the potency of the drug as well as the dose, duration, and individual sensitivity to the drug. These factors also determine the time of recovery after cessation of the drug.

ACTH Stimulation Test

The ACTH stimulation test is commonly used for diagnosis of HC in dogs. In this test, ACTH is administered at a supraphysiologic dose that should maximally stimulate the release of cortisol from the adrenal glands. In dogs with PDH, the capacity of the adrenal cortices to secrete cortisol is greater than in healthy dogs, and the adrenal cortices are responsive to ACTH. Thus, in the majority of dogs with PDH, the post-ACTH serum cortisol concentrations are higher than those in healthy dogs. In AT, however, the neoplastic cells are not necessarily responsive to ACTH and they secrete cortisol more erratically. Thus, the results of ACTH stimulation in dogs with AT are less predictable: post-ACTH cortisol concentrations can be above, within, or (rarely) below the reference interval. The exact sensitivity of the ACTH stimulation test for diagnosis of HC is debatable and varies substantially between studies, with results of 60% to 95%. It is generally agreed, however, that the sensitivity of the test is low for PDH and even lower for AT.[1,4,13-15] Because of the low sensitivity, a negative result in the ACTH stimulation test should not be used to rule out the diagnosis of HC.

The specificity of the ACTH stimulation test (i.e., 1 − the proportion of false-positives results in dogs that do not have HC) varies depending on the population being tested. In people, false-positive results suggestive of HC have been reported in patients with chronic renal failure, DM, anorexia nervosa, malnutrition, and various critical illnesses.[16] In dogs presented for a variety of diseases (unregulated DM, liver disease, or renal disease) one study found a specificity of 64%.[17] In contrast, dogs with well-controlled DM all had negative ACTH stimulation test results (i.e., 100% specificity).[18] In 2 studies the ACTH stimulation test was evaluated in dogs with recently diagnosed neoplasia.[19,20] Combining the results of these 2 studies together, only 2 of 37 dogs with lymphosarcoma had positive ACTH stimulation test results (i.e., 95% specificity). In a group of dogs with nonhematopoietic neoplasia, 2 of 15 were positive for HC by the ACTH stimulation test (i.e., 87% specificity).[19] In a large study that used necropsy as a gold standard for diagnosis of HC, 4 of 41 dogs had positive ACTH stimulation test results (i.e., 91% specificity).[14] In the largest study that evaluated the specificity of the ACTH stimulation test, in a group of dogs with a variety of nonadrenal diseases, only 8 of 59 dogs were positive by the ACTH stimulation test (i.e., 86% specificity). Importantly, this study showed that false-positive ACTH stimulation test results are more common in dogs that are severely ill compared with dogs with only mild or moderate illness.[13] Thus, appropriate patient selection is imperative when performing the ACTH stimulation test. If the index of suspicion of HC is high and other diseases are appropriately ruled out, the specificity of the ACTH stimulation test is reasonably high with an expected high positive predictive value.

The ACTH stimulation test is the test of choice for diagnosis of iatrogenic HC. Low-normal or diminished results are expected because of decreased capacity of the adrenal cortices to secrete cortisol. The cortisol assay might cross react with the glucocorticoid drug that is causing HC, thus giving a falsely high basal cortisol result. In such a case, however, there should be no significant difference between the baseline concentrations and the post-ACTH concentrations of "cortisol."

Protocol Serum (or plasma) concentrations of cortisol are measured before and 1 hour after an intravenous or intramuscular injection of ACTH (most commonly in the form of a synthetic ACTH analog: Cortrosyn or Synacthen). ACTH

is administered at a dose of at least 5 μg/kg and up to 250 μg/dog.[21] The test can begin at any time of day and without any patient preparation.

Low-dose Dexamethasone Suppression Test

The low-dose dexamethasone suppression test (LDDST) is considered the test of choice for diagnosis of HC because of its relative accuracy.[4,22] In this test, dexamethasone (sodium phosphate or in polyethylene glycol) is administered intravenously at 0.01 to 0.015 mg/kg, a dose that suppresses the secretion of ACTH from a healthy pituitary gland for over 24 hours.[4] Lack of ACTH secretion leads to decreased cortisol concentrations within 2 to 3 hours. Blood for cortisol measurement is collected just before, and at 4 and 8 hours after dexamethasone administration. In normal dogs, serum cortisol concentrations typically fall below 20 nmol/L by 4 hours and remain at that level by 8 hours after dexamethasone administration. In dogs with PDH, the abnormal corticotrophs quickly escape suppression by dexamethasone and resume secretion of ACTH within less than 8 hours, thus stimulating cortisol secretion from the adrenal cortex and leading to increased serum cortisol concentrations and a positive LDDST result (> 40 nmol/L at 8 hours). In chronically ill and stressed dogs, the suppressive effects of dexamethasone can be overridden by the stimulation from the hypothalamus, resulting in a false-positive test result. In dogs with HC caused by an adrenal tumor, cortisol secretion is largely independent of ACTH regulation; suppression of the pituitary gland by dexamethasone has little or no effect on secretion of cortisol and the test results are consistently positive. Measuring cortisol concentrations at 0 and 4 hours is not necessary for interpretation of the test result but is informative in differentiating PDH from AT (discussed below).

The sensitivity of the LDDST is high, varying between 85% and 100% in different reports.[12,23] The reported specificity of the LDDST varies greatly depending on the population being tested. In one report that included critically ill dogs, the specificity was only 44% (i.e., 56% of dogs with diseases other than HC tested positive) with no significant difference in the 8-hour cortisol concentration between dogs with HC and dogs with other diseases.[13] Other authors suggest that the specificity is as high as 95% if dogs are carefully selected based on clinical signs and laboratory test results that are consistent with HC, as described above.[4] Thus, ruling out other diseases before using the LDDST is essential.

Transient stress, even if seemingly minor, should be avoided for the duration of the LDDST. A recent study evaluated the effect of a mock ultrasound scan procedure on the results of an LDDST in healthy young dogs. The procedure included clipping of abdominal hair, positioning in dorsal recumbency, and a 20-minute mock scan in a dark room. This procedure was performed once, during the 8 hours of the LDDST, at 2, 4, 6, or 8 hours. In 1 out of 6 dogs, cortisol concentrations were above the diagnostic cutoff on 2 different occasions just after the mock ultrasound.[24] It is plausible that in dogs with chronic illness that undergo an ultrasound scan, the rate of false-positive results on the LDDST would be higher.

Urine Cortisol:Creatinine Ratio

Urine cortisol measurement is a reflection of the cumulative amount of cortisol excretion over a period of a few hours. The concentration of cortisol in the urine depends on the concentration of the urine. For standardization, the urine cortisol concentration is divided by the concentration of creatinine (which is filtered in the kidneys at a near-constant rate). Assays that measure urine cortisol cross-react differently with various cortisol metabolites that are found in the urine. This leads to considerable variation in the reference interval for the urine cortisol:creatinine ratio (UCCR) between different laboratories. Reference intervals for UCCR should therefore be generated separately by each laboratory and results cannot be directly compared between them.

In contrast to the ACTH stimulation test and the LDDST, the UCCR is not a dynamic test. It does not assess the response of the hypothalamus-pituitary-adrenal gland axis to stimulation or suppression. Thus, the UCCR has poor specificity of about 23%, i.e., it is abnormally high in about 77% of dogs with diseases other than HC.[13,25] This test is valued mainly for its high sensitivity: 75% to 100%.[13,25] Similar in sensitivity to the LDDST, the UCCR is much more convenient and is the test of choice for ruling out HC when the index of suspicion is low.[1] UCCR varies considerably from day to day in dogs.[26] To avoid occasional false negatives, it has been suggested to collect 10 consecutive samples on 10 different mornings.[27] Like the LDDST, the UCCR is affected by transient stress and it is best run on a free-catch urine sample that is collected at home.

Basal Cortisol

Basal serum cortisol, as mentioned before, is not a useful test for diagnosis of HC. It is a common misconception that a high cortisol concentration that is measured as the baseline in the ACTH stimulation test or in the LDDST should increase the suspicion of HC. This is incorrect because stress (from illness, injury, or even just from visiting the clinic) can increase the cortisol concentration above the reference interval. Similarly, basal cortisol is inaccurate in assessing response to medical treatment of HC. Basal cortisol can be used to rule out hypoadrenocorticism caused either by disease[28] or by cortisol suppression related to treatment.[29] If the basal cortisol is not low (i.e., if it is > 55 nmol/L), hypoadrenocorticism is very unlikely.

Oral LDDST and UCCR Combination

This test combines the advantages of the LDDST and UCCR. Like the regular LDDST, it is a dynamic test, assessing the ability of the pituitary-adrenal axis to escape the suppressive effect of dexamethasone. Like the UCCR, it avoids the stresses of hospital environment and restraining

for blood draws. Unfortunately, it also requires an elaborate protocol that is executed entirely by the dog's owners and thus its success is dependent on client compliance.

Protocol Urine is collected by the owner on 2 consecutive days at 08:00 hours for measurement of baseline UCCR. Immediately after collection of the second urine sample, the owner administers an oral dose of dexamethasone, 0.01 mg/kg. After walking the dog at 12:00 and 14:00 hours to ensure bladder emptying, the owner collects a third urine sample from the dog at 16:00 hours.

Preliminary evidence suggests that a 50% decrease in UCCR from the baseline or a UCCR $< 1 \times 10^{-6}$ is consistent with normal suppression in healthy dogs.[30] Dogs with HC are expected to avoid suppression and have a lesser decrease from the baseline value of the UCCR. Unfortunately, the diagnostic accuracy of this test in dogs with HC and in dogs with other diseases is yet to be reported.

ACTH Stimulation and Measurement of 17-alpha-hydroxy Progesterone (17HO-Prog) and Other Sex Hormones

In the zona glomerulosa of the adrenal cortex, cortisol is the end product of a series of enzymatic steps that begin with conversion of cholesterol to pregnenolone and continue through numerous other sex hormones. In the zona reticularis of the adrenal cortex, the end products of a similar chain of enzymes are sex hormones exclusively. In health, only very small amounts of sex hormones are released to the circulation from the adrenal gland, but much larger quantities of cortisol are produced and released. The concept of measuring sex hormones after ACTH stimulation is based on the supposition that in some atypical cases of Cushing's syndrome, the production of cortisol is minimal and excess cortisol precursors/sex hormones cause the clinical signs. Beyond being a misnomer (Cushing's syndrome is defined as hypercortisolism), the existence of atypical Cushing's syndrome is debatable.[31] It is beyond the scope of this review to discuss the evidence for and against the existence of atypical Cushing's syndrome and the reader is referred to a review on the subject.[31] It is important to note, however, that there are only rare reported cases in which the evidence for atypical Cushing's syndrome is substantial.[32,33] Among the various cortisol-precursurs, 17-hydroxy-progesterone has received the most attention as a candidate for measurement in the diagnosis of atypical Cushing's syndrome.[31] Combining the results from various studies, the accuracy of this test is lower than that of the LDDST and/or the ACTH stimulation test with cortisol measurement.[31,34] In the occasional case in which the results of an LDDST and ACTH-stimulated cortisol are negative but the ACTH-stimulated 17-hydroxy-progesterone is high, the suspicion of adrenal disease might be raised. On the other hand, because of its low specificity, the possibility of a false positive on the ACTH-stimulated 17-hydroxy-progesterone is likely in these cases.

Test Selection: Differentiation

Differentiation between PDH and AT is important for determination of prognosis and best treatment plans. Clinical signs and biochemical markers cannot be used to differentiate AT from PDH. Differentiation between PDH and AT can be aided by laboratory results in the majority of cases, but these tests are increasingly replaced by ultrasonography. Ultrasound scans provide more information. They aid in diagnosis and in ruling out other diseases, as well as in differentiation between the different causes of HC.[1,35] A complete review of imaging modalities in the diagnosis of HC is beyond the scope of this review. Here we will discuss laboratory tests that are used for differentiation of PDH from AT: LDDST, high-dose dexamethasone suppression test (HDDST), endogenous ACTH, and UCCR.

Dexamethasone Suppression Tests

The sensitivity of pituitary tumors to the suppressive effects of dexamethasone varies. Depending on the dose of dexamethasone used, over 65% of dogs with PDH will show some degree of cortisol suppression. Suppression of cortisol secretion will not occur in AT because ACTH secretion is already suppressed. Because of fluctuations in cortisol production, however, serum cortisol concentrations in dogs with AT will sometimes appear to be suppressed.

In the LDDST, assuming the 8-hour serum cortisol concentration is above the diagnostic cutoff and the diagnosis of HC is made, suppression is defined as: 1) a cortisol measurement at 4 and/or 8 hours that is <50% of the baseline cortisol; or 2) a cortisol measurement at 4 hours that is below the diagnostic cutoff (< 40 nmol/L).[1] If one or more of those criteria are met, the diagnosis of PDH can be made. If none of those criteria are met, PDH and AT are still plausible and further testing is necessary.

In HDDST, a dose 10 times higher than the LDDST is administered intravenously (0.1 mg/kg) and the protocol of blood sampling is the same.[4] Criteria of suppression in this test are the same as above but also include suppression below the diagnostic cutoff for diagnosis of HC at 8 hours (< 40 nmol/L). Because the dose of dexamethasone is higher, dogs with PDH are more likely to show evidence of suppression; however, they are also likely to have suppressed cortisol concentrations at 8 hours, thus precluding the use of this test for diagnosis of HC. Dogs with AT typically will not show evidence of suppression despite the higher dose of dexamethasone because their ACTH secretion is suppressed to begin with. In PDH, about 75% of dogs will meet at least one criteria of suppression on the HDDST. Thus, the sensitivity of the HDDST for diagnosis of PDH (75%) is only marginally higher than the sensitivity of the LDDST (65%). In a dog that was diagnosed with HC based on an LDDST, if a diagnosis of PDH could not be made based on the LDDST, it would probably be best to use ultrasonography for differentiation. If a diagnosis of HC was made based on an ACTH stimulation test, the HDDST could be considered.

The HDDST can be combined with the UCCR and performed by owners at home. Urine is collected on 2 separate mornings for measurement of UCCR. Starting immediately after collection of the second urine sample, dexamethasone is administered orally at a dose of 0.1 mg/kg and this dose is given 2 more times on that day at 8-hour intervals. A third urine sample is collected on the third morning. A decrease in UCCR to less then 50% of baseline is considered consistent with PDH. Lack of suppression is consistent with PDH or AT.[36] The sensitivity of this test for detection of PDH is similar to the regular HDDST, 72%. If the results are negative, both PDH and AT should still be considered.

Endogenous ACTH

Measurement of endogenous plasma ACTH concentration is the most accurate test for differentiation of PDH from AT. In dogs with AT, ACTH secretion is suppressed and its concentrations in the plasma are invariably low. In PDH, secretion of ACTH is variable, but its plasma concentrations are either normal or high. In a recent study, all dogs with AT had plasma ACTH concentrations below the limit of detection of the assay (< 5 pg/mL), whereas in dogs with PDH, the median plasma ACTH concentration was 30 pg/mL (range, 6-1250 pg/mL). Thus, there was no overlap in ACTH concentrations between dogs with PDH and AT with a suggested 100% accuracy.[37] This study used a new ACTH chemiluminescent immunometric assay (Immulite ACTH). Previous studies used older assays that were less sensitive and demonstrated lesser accuracy with some overlap of ACTH concentrations between PDH and AT.[4]

Endogenous ACTH concentration is not a popular method for differentiation of PDH from AT because it requires special handling of samples. Blood should be collected into a pre-chilled EDTA-coated tube and then immediately centrifuged at 4°C. After separation, the plasma should be transferred into a plastic tube and shipped frozen to the laboratory. ACTH will degrade quickly at room temperature and/or as a result of repeated freezing and thawing. Collecting the sample with the proteinase inhibitor aprotonin decreases the problem of degradation, but the presence of aprotonin causes a negative bias in the assay. Because of the sensitivity of ACTH to degradation, collection and handling of the sample can easily result in falsely decreased concentrations and an incorrect diagnosis of PDH as AT. High ACTH concentrations consistent with PDH are diagnostic, even if a suboptimal protocol has been used for collection and shipping of the sample.

Because ACTH secretion in health is pulsatile and its concentrations overlap between dogs with HC and healthy dogs, it has no value in confirming the diagnosis of HC.

Desmopressin Stimulation Test

Desmopressin (DDAVP) is a synthetic long-acting analog of vasopressin (antidiuretic hormone). It has selective affinity to the V2 and V3 subtypes of the vasopressin receptor that are present in the pituitary gland, and it stimulates ACTH secretion. Such stimulation is unlikely to occur in dogs with AT. Adrenal tumors often express the V1 subtype that is less responsive to DDAVP. Therefore, serum cortisol concentrations are expected to increase in response to DDAVP in dogs with PDH but not in those with AT. A vasopressin stimulation test has been evaluated in the past and was not useful in discriminating PDH from AT.[38,39] Those studies used lysine-vasopressin, which is not selective and activates the V1 receptor in addition to V2 and V3. In contrast, a recent study found that serum cortisol concentrations increased significantly (> 10% of baseline) 30 minutes after stimulation with an intravenous injection of DDAVP (4 μg/dog) in the majority of dogs with PDH and about half of healthy dogs, but in none of the 7 dogs with AT.[40] The test was 87% sensitive and 100% specific for diagnosis of PDH. These results are promising but should be further evaluated in larger studies.

Common Questions and Answers

Which Test Is the Best for Diagnosis of HC? ACTH Stimulation Test or LDDST?

No test is 100% accurate. The choice of a diagnostic test depends on the clinical presentation, cost, convenience, and, importantly, on the purpose of the test. As discussed before, chronic stress of nonadrenal disease might be sufficient to cause a false-positive result on any test for HC. Acute stress, however, is unlikely to affect the ACTH stimulation test. In contrast, the LDDST is relatively sensitive to the effect of acute stress. For dogs that are easily stressed by traveling, being in a hospital environment, or by handling, the ACTH stimulation test might be a better first choice than the LDDST. The ACTH stimulation test is convenient because it takes less time and does not require any special conditions or preparation. It is the only test that can be used to diagnose iatrogenic HC. It is the most specific test for diagnosis of HC and it can be used as a baseline for treatment (although usually such a baseline is unnecessary). It can also be extended to include sex hormones, although the value of measuring sex hormones is questionable. The ACTH stimulation test has poor sensitivity for diagnosis of HC and should not be used to rule it out. It is probably not an efficient choice in a patient that is very likely to have HC because in about one third of dogs with HC it will yield a false-negative result requiring a second test. The LDDST is considered to be the most accurate test for diagnosis of HC and at least in the setting of a referral practice it seems to be the most popular test.[22] It is only accurate, however, if a thorough diagnostic workup precedes it and excludes other potential diseases. In cases in which another disease process has been diagnosed and HC is suspected, either as a predisposing factor (diabetes, gallbladder mucocele, urinary tract infection) or as a concurrent disease, it is best to treat the other disease process to the extent possible and then to test for HC. If treatment is unsuccessful and HC is suspected to be the reason, the ACTH stimulation test should be used.

Not all owners report that their pet is receiving a drug either because they do not realize that their pet is exposed to the drug or they do not understand that a drug is a drug (e.g., over-the-counter drugs). This should be considered when a patient is presented with signs of HC without apparent exposure to glucocorticoid drugs. If an LDDST is performed and the results are negative, iatrogenic HC should be suspected and an ACTH stimulation test performed. If an LDDST is performed and the results are positive, an ACTH stimulation should be considered if the degree of clinical suspicion of HC is not high and a false positive is a possibility. Before deciding on a test, the goal of the long-term plan should be considered. If testing for HC is discussed with the owners and differentiation between AT and PDH is not desired by the owners, the LDDST becomes a less attractive test.

Can the ACTH Stimulation Test Be Used in a Dog That Is Currently on a Glucocorticoid Drug?

Yes, depending on the purpose of the test. The test is affected by the drug in 2 ways. First, some glucocotricoid drugs like prednisone and its derivatives cross-react with the cortisol assay. Others, like dexamethasone, do not. Cross-reactivities of different glucocorticoid drugs with cortisol assay on the Immulite 2000 (a chemiluminescent enzyme immunoassay by Siemens) are presented in Table 4. This assay is widely used worldwide in reference laboratories. Second, chronic administration of glucocorticoids will cause suppression of the pituitary-adrenal axis, with individual response varying considerably. Typically, the recommendation is to withdraw any steroid drug before performing the ACTH stimulation test so that the exogenous steroid would not cross-react with the assay. The duration of withdrawal that is required depends on the drug with some glucocorticoids persisting in the body for many weeks. If discontinuation of treatment is not possible, or if the test is performed to rule out decreased function of the adrenal glands (either caused by iatrogenic HC or by Addison's disease), it can be performed even in the presence of exogenous steroids. An increase in cortisol concentrations after ACTH stimulation indicates that the adrenal glands are functioning. If no stimulation is seen, and both the pre-ACTH and post-ACTH cortisol amounts are normal, it is likely that the adrenal glands are suppressed and the assay is cross-reacting with an exogenous steroid. This is because the concentrations of the drug are unlikely to change significantly during the 1 hour that the test lasts. If no stimulation is seen, and both the pre-ACTH and post-ACTH cortisol amounts are low, the results are consistent with iatrogenic HC and with Addison's disease. Typically, in dogs that are receiving steroid drugs or were recently receiving them, the basal cortisol is low to low normal and a small increase is seen after ACTH stimulation.

Table 4. Cross-reactivities of Various Drugs with the Cortisol Assay on the Immulite 2000

Drug	Cross Reactivity (%)
Prednisolone	62
Methylprednisolone	25
Prednisone	6
Cortisone	1
Triamcinolone	0.02
Dexamethasone	Not Detected
Betamethasone	Not Detected
Fluticasone	Not Detected
Progesterone	Not Detected
Spironolactone	Not Detected
Tetrahydrocortisone	Not Detected

In a Dog with Diabetes, What Is the Best Way to Diagnose HC?

In a diabetic dog, all the clinical signs and laboratory test abnormalities present in DM can be a manifestation of HC except for the presence of cataracts.[41] It is therefore common to suspect HC in diabetic dogs, especially when insulin therapy is unsuccessful. Dogs with HC have some abnormalities that are not seen in DM: truncal obesity, panting, symmetrical alopecia, comedones, calcinosis cutis, and reproductive abnormalities. The presence of these physical abnormalities in a diabetic dog should raise the suspicion of HC. On the other hand, there are no clinicopathological findings that suggest a diabetic dog has HC concurrently as opposed to DM alone. It is not uncommon for practitioners to screen diabetic dogs for HC when liver enzymes are markedly elevated or when there is severe hyperlipidemia, but these changes, like others, are not specific for HC.

The suspicion of HC is increased when insulin therapy is unsuccessful. Although HC leads to insulin resistance, it is just one of many possible causes of failed insulin therapy.[41,42] If clinical signs that are specifically suggestive of HC are not present (as described above), one should first rule out other reasons for poor response to insulin. Owner's compliance, technique of administration, and other problems related to the insulin therapy, as well as urinary tract infections, pancreatitis, and other diseases, should be investigated first. If insulin resistance (typically more than 1.0-1.5 U/kg twice per day) is still suspected, after other causes have been eliminated, the diagnosis of HC should be perused.

When choosing a dynamic endocrine test for diagnosis of HC in a diabetic dog, the most specific test should be chosen. This is because the clinical suspicion is already very high, but the effect of chronic stress is likely to cause a false positive. In this situation, a negative result for HC, on any of the endocrine tests, is highly suggestive that the dog does not have the HC. A positive result on any of the tests should be interpreted cautiously. Because a false positive is likely, an ACTH stimulation is probably the most appropriate as a first choice. In contrast to nondiabetic dogs, a negative result (normal response) on an ACTH stimulation test in an unregulated diabetic dog is likely a true negative. Well-regulated diabetics

have been shown to have normal responses on the ACTH stimulation test, but in other studies the severity of concurrent illness (e.g., DM) has been correlated to the rate of false positive on the ACTH stimulation test.[16,18]

In a Dog with Elevated Liver Enzymes, How Do I Differentiate Liver Disease from Cushing's Syndrome?

Differentiating between primary liver disease and HC can be challenging in mild cases. Taking a thorough history regarding appetite, activity level, general attitude, and duration of clinical signs is crucial. Clinical signs that overlap include Pu/Pd, pendulous abdomen, and hepatomegaly. Although these signs are commonly seen as the only clinical signs in dogs presented for HC, they are infrequently present without other signs in dogs with primary liver diseases. Typically, dogs that have liver disease and Pu/Pd as a dominant sign would have some degree of ascites, which is not a consequence of HC.

Laboratory tests that overlap include increased liver enzyme activities, increased serum bile acid concentrations, and decreased serum urea concentrations. If other laboratory abnormalities that are consistent with liver disease are present, Cushing's syndrome should not be suspected. For example, hyperbilirubinemia, hypoalbuminemia, microcytosis, or abnormal clotting times should sway the clinician away from screening for HC.

If there is a complete overlap in clinical signs and laboratory results, screening tests for Cushing's syndrome are likely to be unhelpful in differentiating the 2 diseases. A negative test result for HC on the UCCR or the LDDST can be used to rule it out with confidence. The high rate of false-negative ACTH stimulation test results precludes using it to rule out HC. A positive result on either one of the three might be a false positive associated with stress related to a primary liver disease. In this scenario, imaging and biopsy of the liver are essential.

Differentiation of Hypothyroidism and Cushing's Syndrome

Dogs presented for alopecia, lethargy, and weight gain are often tested for HC and hypothyroid at the same time. Hyperlipidemia and a mild increase in ALP and alanine aminotransferase activities can be seen in both diseases. Hypothyroidism is associated with decreased appetite, generalized weight gain, and mild anemia, signs that should sway the clinician away from suspecting HC. Also, the classic signs of HC—polyphagia and Pu/Pd—should sway the clinician from suspecting hypothyroidism. In cases in which clinical and clinicopathological signs overlap and there are no other signs to suggest nonthyroidal disease, HC should be tested first and only if the results are convincingly negative, hypothyroidism should be considered. This is because T4 and free T4 are decreased in up to 57% and 64% of dogs with HC, respectively. TSH is typically decreased as well. In contrast, there are no reports of hypothyroidism causing false-positive results on tests of the pituitary-adrenal axis (at least not beyond the normal rate of FP).

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