Biological Variability in Serum Cortisol Concentration
Post-adrenocorticotropin Hormone Stimulation in Healthy Dogs

A. Gal, K. Weidgraaf, J.P. Bowden, N. Lopez-Villalobos, N.J. Cave, J.P. Chambers, and F. Castillo-Alcala

Background: The ACTH stimulation has low sensitivity for the diagnosis of hypercortisolism possibly as a result of biological and analytical variability.

Hypothosis/Objectives: To report the components of biological and analytical variability in serum cortisol concentration post-ACTH stimulation ([cortisol]) in healthy dogs.

Animals: Fourteen healthy harrier hound dogs.

Methods: The data were extracted from a separate, prospective, randomized, double-blinded, controlled discovery study in which dogs treated with vehicle control and 4 different doses of cortisone acetate (CA) for 7 days had an ACTH stimulation test performed to confirm the dose-dependent effect of CA. The index of individuality (IoI), the critical difference between sequential measurements (CD), and the number of measurements required to assess the homeostatic set point (HSP) of [cortisol] with confidence intervals (CI) of 90 and 95% were estimated.

Results: The IoI was equal to 1.1 and the CD was 3.3 μg/dL (92 nmol/L). The number of measurements required to assess the HSP of [cortisol] with CI of 90 and 95% were 3 and 15, respectively. Additionally, mean [cortisol] was higher in males than in females (13.3 ± 4 μg/dL [366 ± 114 nmol/L] vs. 11.5 ± 2.5 μg/dL [318 ± 65 nmol/L], respectively; P = .046). As expected, treatment with CA resulted in a dose-dependent suppression of [cortisol].

Conclusions and Clinical Importance: False-negative test results in hypercortisolism could occur when [cortisol] is outside of the individual’s HSP and within the reference interval. The large CD emphasizes the importance of assessing clinically relevant parameters in the diagnosis and monitoring of HC.

Key words: ACTH stimulation test; Canine; Cortisol; Critical difference; Index of individuality.

Abbreviations:
[cortisol] serum cortisol post-ACTH stimulation
ACTH adrenocorticotropic hormone
CA cortisone acetate
CD critical difference between sequential measurements
CI confidence intervals
CV coefficient of variation
HC hypercortisolism
HSP homeostatic set point
IoI index of individuality

Hypercortisolism (HC) is a common endocrinopathy in dogs resulting from excessive cortisol production by the adrenal glands and has an estimated incidence of 1–2 cases per 1,000 dogs per year.1 Veterinarians base the diagnosis of HC on supportive clinical signs, clinicopathologic changes, and specialized endocrine tests.2 A recent consensus statement from the American College of Veterinary Internal Medicine indicated that the diagnosis of HC depends on the demonstration of either increased cortisol production or decreased sensitivity of the hypothalamic-pituitary-adrenal axis to negative glucocorticoid feedback.2 The adrenocorticotropic hormone (ACTH) stimulation test is one of the specialized endocrine screening tests for HC that works by assessment of the adrenocortical reserve.2 The test’s wide ranges of sensitivity and specificity for all forms of spontaneous HC in dogs lead to false-negative and false-positive test results and uncertainty in the interpretation of the test.2 Therefore, the clinician sometimes faces difficulty in arriving at a definitive diagnosis of HC. Additionally, monitoring the concentration of serum cortisol concentration ([cortisol]) post-ACTH stimulation is an established method of assessing response to treatment with mitotane or tri-lrostone, and [cortisol] has been used by some to classify the response to treatment as “excessive,” “ideal,” “acceptable,” or “inadequate.”3

The total variability of a diagnostic test affects its performance and influences the way the clinician should use the test. The total variability consists of the components of within- and between-individual biological variability, and the component of analytical variability.4,5 The components of total variability determine the number of repeated measurement required for estimation of the homeostatic set point (HSP) for a diagnostic test parameter, which is the mean and confidence intervals (CI) of the test parameter. The components of total variability also determine the index of individuality (IoI) of a test parameter, which reflects the relationship between the within- and between-individual variabilities for that parameter. A test parameter that has a low IoI

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(Fig 1, test B) would have a low within-individual variability in comparison with its between-individual variability. Tests with low IoI are not well suited for use in population-based reference intervals because the individual’s HSP covers only a small fraction of the population reference interval. Therefore, a test result that is outside of an individual’s HSP, but within the population reference interval, would be erroneously considered normal. Moreover, the critical difference between sequential measurements of a test parameter \( CD \) is the difference not due to the components of biological and analytical variability. Without the \( CD \), the clinician will not be able to tell whether sequential test results are substantially different or whether the difference between them is due to the total variability in the test.

In this study, we report the IoI and \( CD \) of serum cortisol concentration after stimulation with ACTH in healthy dogs, as well as the number of measurements required to assess the HSP of [cortisol] for an individual. Data on repeated measurement of [cortisol] in 14 dogs were available for analysis from a separate discovery study that was designed to detect cortisol metabolites in the urine. We used the ACTH stimulation test to confirm that cortisone acetate (CA) had a biological effect by demonstrating a dose-dependent effect of CA.

**Materials and Methods**

**Animals**

We used data collected from 14 healthy harrier hound dogs (8 intact females, 1 spayed female, 1 intact male, and 4 castrated males) from the Massey University dog colony. The mean ± standard error (±SD) age of the dogs was 7.1 ± 3.4 years. The mean (±SD) male age was 6.0 ± 5.6 years and the mean (±SD) female age was 7.7 ± 3.7 years. The mean (±SD) body weight during the period of the study ranged from 26.4 ± 2.5 kg to 26.7 ± 2.5 kg (Fig S1). Mean (±SD) male body weight throughout the study was 29.1 ± 1.7 kg and mean (±SD) female body weight throughout the study was 25.0 ± 1.7 kg. The dogs had been in the colony for at least 6 months before commencement of the research and were habituated to the environment and personnel, which remained unchanged throughout the duration of the study. Dogs were housed in pairs, in concrete floor pens each comprising an indoor kennel (2.9 m × 5.5 m), with continual free access to an outdoors concrete area (2.4 m × 2.8 m). Dogs had free outdoor access in fenced, grassed areas for at least 2 hour each day. The dogs consumed the same diet for at least 6 months before the study’s commencement which was a mixture of a high moisture canned diet, and an extruded kibble. A board-certified clinical nutritionist (NC) formulated both diets to meet the nutritional requirements for maintenance, as established by the Association of American Feed Control Officials. Water was provided ad libitum.

**Study Design**

The Massey University Animal Ethics Committee approved this study (protocol #15/07). The study was prospective, randomized, double-blinded, and controlled. The purpose of the discovery study was to detect cortisol metabolites in the urine. During the study, all dogs received all levels of treatments in a random sequence (i.e, a Latin Square design) and both, the dog handlers and the authors, were blinded to the treatment level (Fig S2). A compounding pharmacy prepared the microcrystalline cellulose vehicle control and the different doses of CA in 1 batch. The planned dosages were 0.5 mg/kg, 1 mg/kg, 1.5 mg/kg, and 2 mg/kg of CA. The actual dosages ± standard deviation (SD) were 0.48 ± 0.1 mg/kg, 0.95 ± 0.1 mg/kg, 1.43 ± 0.2 mg/kg, and 1.90 ± 0.2 mg/kg of CA when considering the range of body weights of the dogs (Fig S2). The dog handlers dosed the dogs once a day, PO, with vehicle control or CA. The doses of CA were approximately 0.5× to 2× the physiological range of cortisol.

**Fig 1.** Illustration of 2 hypothetical diagnostic tests with high and low IoI, respectively. Test A has high IoI and has large within-individual variability and small between-individual variability. Each of test A’s within-individual variabilities covers most of the population-based reference interval. Test B has low IoI and has large between-individual variability and small within-individual variability. Each of test B’s within-individual variabilities covers a small fraction of the population-based reference interval. Test B, with the low IoI, is not suited for interpretation by the population-based reference interval. IoI, index of individuality; double-dashed line, entire population-based reference interval; horizontal line with a dot in the center, each dog’s test’s mean and confidence interval for the mean.
Dosing took place between 0800 and 1000 for 7 days in each of the 5 treatment periods, and a washout period of 14 days separated each treatment period. The length of washout period was based on previous literature and was judged to be sufficient to prevent a carry-over effect of treatment from 1 treatment period to the next.7–9 An ACTH stimulation test was performed 24 hour after the last dose of CA for each of the 5 treatment periods. Cortisone acetate has a reported elimination half-life of 23 minutes;8 hence, CA was not present in the dogs’ serum 24 hour after the dogs’ last doses of CA, when the ACTH stimulation test was performed. A washout period of 24 hour between the discontinuation of CA and measurement of urine cortisol-to-creatinine ratio is routinely used in the follow-up of hypophysectomized dogs with pituitary-dependent hypercortisolism.10 The dog handlers recorded the dogs’ body weights at the beginning of each of the treatment periods.

**ACTH Stimulation Test and Determination of Serum Cortisol Concentration**

For the purpose of the ACTH stimulation test, the investigators collected paired whole-blood samples by jugular venipuncture before and 1 hour after the IV injection of 5 μg/kg tetracosactrin into the cephalic vein. The investigators submitted the samples to a commercial veterinary reference diagnostic laboratory7 within 1 hour of collection of the last serum sample. Serum cortisol concentration was analyzed by an electrochemiluminescence immunoassay according to the manufacturer’s instructions after serum separation by centrifugation at 3,000 g for 15 minutes. The cortisol assay has lower and upper limits of measurement of 0.02 and 63.43 μg/dL (0.5 and 1750 nmol/L), respectively, and 0.3% cross-reactivity with cortisone.11 The reference laboratory validated the test for use in dogs (data not shown).

**Statistical Analysis**

The data was analyzed using statistical analysis software.7 The dependent variable, serum cortisol concentration post-ACTH stimulation ([cortisol]), was analyzed with the MIXED procedure using a linear mixed model for repeated measures. The model included the fixed effects of treatment, week of treatment, week of treatment-by-treatment interaction, sex, age, and weight of dog, and the random effect of dog (between-individual variation; Var between). The repeated measures on the same dog were modeled with a compound symmetry error structure to provide the variance components of the total variance of [cortisol] were estimated from the formula

\[ CV_{\text{analytic}} = \frac{1}{2} \left( \frac{\text{SD}_{\text{within}}}{\text{mean}} \right) \]

The number of specimens that should be assayed to be X% confident of achieving an estimate of the HSP within D% of an individual dog was calculated from the formula

\[ n = \frac{Z_x^2 \left( CV_{\text{within}} + CV_{\text{analytic}}^2 \right)}{D^2} \]

The CI of the ACTH Stimulation Test

Treatment resulted in an expected dose-dependent decrease in mean (±SD) serum cortisol concentration ([cortisol]) post-ACTH stimulation (vehicle control, 13.2 ± 1.9 μg/dL [365 ± 49 nmol/L]; 0.5 mg/kg CA, 12.6 ± 1.9 μg/dL [347 ± 49 nmol/L]; 1 mg/kg CA, 12.1 ± 1.9 μg/dL [335 ± 49 nmol/L]; 1.5 mg/kg CA, 11.8 ± 1.9 μg/dL [326 ± 49 nmol/L]; 2 mg/kg CA, 11.2 ± 1.9 μg/dL [308 ± 49 nmol/L]; Fig S3). There was no difference in baseline serum cortisol concentration before ACTH stimulation between the groups (P = .37). Increasing the dose of CA did not significantly decrease the heterogeneity of the variance in [cortisol] (Levin test, P = .08).

We tested the effect of the covariates sex, body weight, age, and period of treatment on [cortisol]. We found that the mean (±SD) [cortisol] was significantly higher for males than for females (13.3 ± 4 μg/dL [366 ± 114 nmol/L] vs. 11.5 ± 2.5 μg/dL [318 ± 65 nmol/L], respectively; P = .046). The other covariates did not have a significant effect on [cortisol].

The means and 90% CI for [cortisol] for each of the dogs and the group mean with respect to the reference laboratory population-based reference interval are presented in Figure 2. The 90% CI for the mean in each dog did not cover most of the population-based reference interval and were unevenly dispersed across the population-based reference interval (Fig 2). The results of the mean, %CV between, %CV within, %CV analytic used to determine whether the level of treatment had an effect on the variation of [cortisol].

![Graph](image-url)
The results of the mean, %CV between, %CV within, %CV analytic, Var between, Var within, Var analytic, Cp, CD %mean, and IoI for serum cortisol post-ACTH stimulation, and the numbers of measurements required to assess the homeostatic set point of serum cortisol post-ACTH with confidence intervals of 90 and 95%.

| Parameter | Result | Units |
|-----------|--------|-------|
| Mean      | 12.2   | µg/dL |
| %CV between | 9     |       |
| %CV within  | 5     |       |
| %CV analytic | 8     |       |
| Var between | 33.1  | µg/dL |
| Var within  | 10.8  | µg/dL |
| Var analytic | 28.9  | µg/dL |
| CD         | 3.3   | µg/dL |
| Cp %mean   | 27.3  |       |
| IoI        | 1.10  |       |
| CI 90%     | 3     |       |
| CI 95%     | 15    |       |

CV between, Var within, Var analytic, Cp, CD %mean, and IoI for [cortisol] and the numbers of measurements required to assess the HSP of [cortisol] with CI of 90 and 95% are summarized in Table 1. The between-individual, within-individual, and analytical components of variability contributed 45, 15, and 40% to the total variability of [cortisol], respectively. Additionally, we found a CD > 3.3 µg/dL (92 nmol/L) to be substantial and not the result of biological or analytical variability. Furthermore, 3 and 15 sequential measurements were required to determine the individual’s HSP of [cortisol] with 90 and 95% CI, respectively. Finally, we calculated an IoI value of 1.1 for [cortisol].

### Discussion

We found that the ACTH stimulation test had an intermediate IoI value of 1.1 for [cortisol] post-ACTH stimulation. Application of a population-based reference interval can have a high level of discrimination between normal and abnormal (Fig 1) wherein a test parameter has an IoI > 1.4, whereas the population-based reference interval should not be used when a test parameter has an IoI < 0.6.\(^2\) The intermediate IoI value of 1.1 for the ACTH stimulation test indicates that the ACTH stimulation test is suitable for use with population-based reference intervals. However, the intermediate value of the IoI also reflects the reported uncertainty in the diagnosis of HC.\(^2\) as exemplified below.

We estimated the HSP for [cortisol] for each dog by calculation of the mean and 90% CI from 5 ACTH stimulation tests. This number of ACTH stimulation tests was sufficient to calculate the HSP for [cortisol] with 90% CI (Table 1). For all dogs in the study, the 90% CI did not approach the upper end of the reference interval. Hence, for all dogs in the study, if a [cortisol] in the upper part of the reference interval had been found, it should have been considered abnormally high, because it exceeded the HSP’s 90% CI, even though it fell within the reference interval. However, because in most cases when the ACTH stimulation test is performed, the HSP of [cortisol] is not known for that individual dog, the results may be incorrectly interpreted to be normal when it actually is abnormally high for that dog (Fig 2). Hence, biological and analytical variability in the ACTH stimulation test may lead to false-negative test results when using the ACTH stimulation test to diagnose HC in dogs.

One way to increase the IoI is to decrease the interindividual variability by stratification of the reference intervals by age, body weight, sex, neuter status, and breed. In our study, “sex” significantly affected [cortisol]. Thus, establishing sex-based reference intervals might improve the accuracy of the ACTH stimulation test. One of the limitations of our study was lack of balance with regard to “breed” and “neuter status.” Future studies should assess the effect of “breed” and “neuter status” on [cortisol]. Similar to “sex,” breed-specific or neuter status-specific population-based reference intervals, or generation of correction factors for 1 population-based reference interval would decrease the between-individual variability and increase the IoI.

A second point for consideration is an aspect of why using the results of the ACTH stimulation test to characterize the clinical response to treatment could be challenging. We found that only a CD > 3.3 µg/dL (92 nmol/L) was substantial and not due to biological or analytical variability. For example, a recently used classification\(^3\) assumed the following clinical classes for [cortisol]: “excessively treated” <1.1 µg/dL (30 nmol/L), “ideal control” 1.1–5.4 µg/dL (30–150 nmol/L), “acceptable control” >5.4–9.0 µg/dL (>150–250 nmol/L), and “inadequate control” >9.0 µg/dL (>250 nmol/L). Accordingly, a clinically well-controlled cortisol of 4.3 µg/dL (120 nmol/L) can be classified as being “excessively treated,” or with “ideal” or “acceptable” control if the second visit test result is within 1.0–7.7 µg/dL (28–212 nmol/L) where in reality, this change in [cortisol] could be due to biological and analytical variability. Hence in this scenario, it would have been beneficial to know the HSP of [cortisol]. A change <CD then would be regarded as clinically relevant if it was outside the CI of patient’s HSP. However, this is not practical because veterinarians will not have ACTH stimulation test results on healthy dogs to serve as a comparison when those dogs are later tested for suspicion of HC.

We note that the calculated CD in our study might not apply to dogs treated with mitotane or trilostane. The CD may not apply because the dogs in our study were not treated with mitotane or trilostane and had [cortisol] that exceeded the current recommendation for clinically controlled treated dogs.\(^3\) Nevertheless, a counter-argument would be that the ultimate goal of
treatment with mitotane or trilostane is to use the lowest dose that will cause adrenal suppression and lead to alleviation of clinical signs of HC, similar to the healthy dogs in our study. Additionally, the analytical component of the total variability is not affected by treatment. Moreover, we suspect that the within-individual and between-individual variabilities would not be affected by treatment. This is because the biological variability in [cortisol] in people is due to variabilities in factors such as the hypothalamus and pituitary. However, there are processes that lead to increased hepatic cortisol inactivation with subsequent overactivity of the hypothalamic-pituitary-adrenal axis, variation in the individual hypothalamic-pituitary variability in [cortisol]. The CV analytic should be a component of the total variability and the number of measurements required to reach the HSP for [cortisol] with 90% and 95% CI. In that respect, it should be taken into consideration that the doses of CA were 0.5 × 2 × the physiological range (the highest dose of CA is as potent as a 0.5 mg/kg dosage of prednisone) and induced minimal suppression (Fig S3). Endogenous ACTH concentration could have provided additional information about the effect of CA on the hypothalamic-pituitary-adrenal axis, but we did not measure it.

The second limitation of the study was the large component of analytical variability. The CV analytic should be <1/2 CV within and in our study, it was > CV within. One possible reason is that the serum samples of some dogs were lipemic, and lipemia can cause interference with the cortisol assay and decrease its precision. An increase in the magnitude of the component of analytical variability can skew the results by increasing IoI, CD, and the number of measurements required to reach the HSP for [cortisol] with 90 and 95% CI. However, in clinical settings, serum is occasionally lipemic, and therefore, our results are relevant to a clinical setting.

In conclusion, the ACTH test has an intermediate individuality that may account for false-negative test results in dogs with HC. Hence, we demonstrated the limitation of the ACTH stimulation test and therefore emphasize the importance of assessing clinically relevant parameters when trying to diagnose HC. We found that CD should be >3.3 μg/dL (92 nmol/L) to assure that the difference between sequential measurements is not due to biological or analytical variability.

Footnotes

| a | Casserole Beef variety; Unicharm Corporation, Minato-ku, Tokyo, Japan |
| b | Pedigree Vital Working Dog with real Beef, Mars, Auckland, New Zealand |
| c | Synacthen® 250 μg/mL, Novartis, NSW, Australia |
| d | IDEXX, New Zealand |
| e | Cortisol assay REF 11875116, Cobas®, Roche |
| f | Statistical Analysis System software, version [9.3] Copyright © 2011, SAS Institute Inc. |

Authors’ contribution

A Gal- formulated the hypothesis, study design, participated in sample collection, analysis of data, and manuscript preparation. F Castillo-Alcala- contributed to study design, sample processing, and manuscript preparation. K Weidgraaf and JP Bowden- participated in animal handling, animal dosing, and sample collection. N Lopez-Villalobos- performed the statistical analyses. NJ Cave and JP Chambers- contributed to study design and manuscript preparation.

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Conflict of Interest Declaration: None of the authors of this manuscript have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the manuscript.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig S1. Dogs’ body weight during the study period. (A) Graphical presentation of the distribution of body weight per dog per week. (B) Mean (±SD) dogs’ body weight per week.

Fig S2. Study design. Dogs were prospectively randomized to five levels of treatment via Latin Square design. Each of the five dosing periods was followed by a 14-day washout period. In each treatment period, the dogs were dosed orally between 0800 and 1,000 for 7 days. The ACTH stimulation test was performed 24 hour after the last dosing for each of the treatment periods. CA, cortisone acetate; W, week.

Fig S3. Summary of the results of serum cortisol concentration (mean ± SD) 1 h after intravenous administration of 5 μg/kg ACTH for each of the five levels of treatment. Groups with different letters are statistically significantly different (P < 0.05).