Comparison of methods to monitor dogs with hypercortisolism treated with trilostane

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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

Abbreviations: 95% CI, 95% confidence interval; γGT, gamma-glutamyl transferase; ACTHst, adrenocorticotropic hormone stimulation test; ADH, adrenal-dependent hypercortisolism; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AUC, area under the receiver operating characteristic curve; b, regression coefficient; CT, computed tomography; day-of-re-evaluation CUCCR, chemiluminescence urine cortisol : creatinine ratio of the re-evaluation day; day-before CUCCR, chemiluminescence urine cortisol : creatinine ratio of the day before the re-evaluation; day-of-re-evaluation LUCCR, liquid chromatography-tandem mass spectrometry urine cortisol : creatinine ratio of the re-evaluation day; day-before LUCCR, liquid chromatography-tandem mass spectrometry urine cortisol : creatinine ratio of the day before the re-evaluation; day-of-re-evaluation USG, urine specific gravity of the re-evaluation day; day-before USG, urine specific gravity of the re-evaluation day; eACTH, plasma endogenous adrenocorticotropic hormone concentration; HC, hypercortisolism; Hp, haptoglobin; IQR, interquartile range; LC-MS/MS, liquid chromatography-mass spectrometry; MRI, magnetic resonance imaging; OR, odds ratio; PDH, pituitary-dependent hypercortisolism; post-ACTH, post-ACTH administration serum cortisol concentration; pre-ACTH, before ACTH administration cortisol concentration; pre pill, serum cortisol concentration before trilostane administration; prepill/eACTH, prepill/eACTH ratio; ROC curve, receiver operating characteristic curve; UCCR, urine cortisol : creatinine ratio; USG, urine specific gravity.
1 | INTRODUCTION

Naturally occurring Cushing's syndrome or hypercortisolism (HC) is a common endocrinopathy in dogs caused by chronic excessive glucocorticoid activity. Trilostane has been the medical treatment of choice for pituitary and adrenal-dependent hypercortisolism (ADH) in the past 20 years. The drug is a competitive inhibitor of the 3β-hydroxysteroid dehydrogenase/isomerase system required to synthesize cortisol, aldosterone, and androstenedione. The appropriate dose and frequency of administration allow trilostane to control the clinical signs and the clinical-pathological abnormalities associated with HC. For several years, the adrenocorticotropic hormone stimulation test (ACTHst) has been used to monitor trilostane treatment. However, over time, concerns have been raised regarding the reliability of this test. The ACTHst has never been validated for trilostane monitoring purposes and the results strictly depend on the time of trilostane administration. Recent evidence has supported a lack of correlation between post-ACTH administration serum cortisol concentration (post-ACTH) and clinical signs.

For these reasons, during the last decade, several methods to monitor trilostane treatment have been investigated. 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. In particular, prepill showed a better correlation with the clinical picture in comparison with post-ACTH. However, when measuring 2 prepill taken an hour apart results significantly differ, thus questioning the ability of this method to replace the post-ACTH. Finally, in 2020, different monitoring variables, such as USG, 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. In the study, none of the previously cited variables was able to differentiate between well and undercontrolled dogs. Haptoglobin concentration (Hp), a moderate acute phase protein, is higher in hypercortisolemic dogs. Haptoglobin concentrations decline during trilostane treatment, suggesting a role of these variables as a monitoring tool to correctly identify trilostane treatment control.

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.

2 | MATERIALS AND METHODS

2.1 | 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.

2.2 | 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 (low-dose dexamethasone suppression test and ACTHst), were enrolled in
the study. 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 postdexamethasone suppression below 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 imaging (MRI) or computed tomography (CT; pituitary height-to-brain value > 0.31 x 10^-2 mm^-3), or ultrasonographically observed symmetrical normal-sized or enlarged adrenal glands (width > 7.5 mm when not available breed-specific cutoff). 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.

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.

2.3 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. 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. 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).

2.4 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). 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-to-day variability. The owner was asked to keep the urine of the day before in the refrigerator until the re-evaluation day.

2.5 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.

2.6 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. 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.22,23 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, v/v) solution containing the deuterated internal standard creatinine-D3. 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+).

2.7 | 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.

2.8 | 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.

3 | 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.

3.1 | 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.
Median and interquartile of time between each re-evaluation (IQR, 7.1-15.1). Nineteen mixed breed dogs, 5 Maltese, 4 Poodles, 3 each Dachshund, Shih-Tzu, Yorkshire Terrier, and 1 each of Riesenshnauser, 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 re-evaluations, and 3 had 6 re-evaluations. The time between each re-evaluation (weeks) is reported in Table 1. The prepill, pre-ACTH, post-ACTH, eACTH, prepill/eACTH ratio (prepill/eACTH), Hp, ALT, ALP, UCCR of the re-evaluation day obtained using chemiluminescence (day-of-re-evaluation UCCR) 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 UCCR) 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-re-evaluation UCCR) was measured in 76 tests. LC-MS/MS UCCR of the day before (day-before UCCR) was measured in 63 tests.

3.2 | Clinical evaluation

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).

3.3 | Association between monitoring methods and total score

Simple and adjusted associations between monitoring methods and owner’s score are reported in Table 2. In multiple regression analysis.
analyses adjusted for trilostane dosage, number of previous re-evaluations, 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.

### 3.4 Association between monitoring methods and inadequate control

Simple and adjusted associations between monitoring methods and inadequate control are reported in Table 3. In multiple regression analysis adjusted for confounding factors serum Hp

| Variables                      | Simple associations | Adjusted associations |
|--------------------------------|---------------------|-----------------------|
|                                | 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       |
| $\gamma$ GT                    | 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 LUCCRa    | 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; $\gamma$GT, 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 LUCCRa, liquid chromatography-tandem mass spectrometry urine cortisol : creatinine ratio 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.

*Available for 76/94 observations.

| Variables                      | AUC      | 95% CI     | Cutoff | Specificity % | Sensitivity % | Accuracy % |
|--------------------------------|----------|------------|--------|---------------|---------------|------------|
| ALT (U/L)                      | 0.76     | 0.66-0.86  | 120    | 90            | 56.3          | 67         |
| $\gamma$ GT (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 LUCCRa    | 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 $\geq$ 0.75 are reported; in bold, AUC $\geq$ 0.75.

Abbreviations: 95% CI, 95% confidence interval; $\gamma$GT, 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 LUCCRa, 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.

*Available for 76/94 observations.
**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

| Variables                        | Simple associations | Adjusted associations |
|----------------------------------|---------------------|-----------------------|
|                                  | 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<sup>a</sup> | 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, before ACTH administration serum cortisol concentration; prepill cortisol, before trilostane administration serum cortisol concentration; prepill/eACTH, prepill/eACTH ratio.

<sup>a</sup>Available for 37/43 observations.

(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, day-of-re-evaluation LUCCR, pre-ACTH, and post-ACTH did not reach statistical significance. The AUC of the variables is reported in Table 4. 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).

### 3.5 Clinical score predictive model

In the multiple regression analysis, Hp was the best predictor of the clinical score ($R^2 = 0.359$). 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 ($R^2 = 0.382$). No other variables had a significant added value.

### 3.6 Sensitivity analysis

Forty-three dogs were included in the sensitivity analysis at the first re-evaluation after diagnosis. Simple and adjusted associations
**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

**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 CUCR  | 84 (42-129)             | 90 (112-774)             | .24     |
| Day-of-re-evaluation LUCCR | 4.1 (2.6-9.1)           | 4 (2.9-6.9)              | .67     |
| Prepill cortisol (μg/dL)   | 2.6 (2.4-4.2)           | 3.6 (2.4-5.5)            | .24     |
| (nmol/L)                   | 71.7 (55.2-115.9)       | 99.3 (66.2-151.7)        |         |
| Pre-ACTH cortisol (μg/dL)  | 2.7 (2-3.5)             | 2.5 (1.9-4.9)            | .74     |
| (nmol/L)                   | 74.5 (55.2-96.6)        | 69 (52.4-135.2)          |         |
| Post-ACTH cortisol (μg/dL) | 4.6 (3.7-6.2)           | 6.2 (3.3-8.8)            | .37     |
| (nmol/L)                   | 126.9 (102.1-71.1)      | 171.1 (91-242.8)         |         |
| 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-CUCR, 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.

*Available for 37/43 observations.
between each monitoring method and the total score are reported in Table 5. 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 under-controlled. The monitoring methods were compared between well-controlled and undercontrolled groups by the Mann-Whitney U test (Table 6). Alanine aminotransferase, γGT, and Hp were significantly higher in undercontrolled dogs (Figures 1-3). All the 3 variables showed a good discriminative ability, and optimal cut-offs were ≥86 U/L, ≥5.8 U/L, and ≥151 mg/dL for ALT, γGT, and Hp, respectively (Table 7).

3.7 | 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 μg/mL and the abdominal ultrasound showed a hyperechoic 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.

3.8 | Low prepill and post-ACTH cortisol results

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 well-controlled 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.

4 | 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.\textsuperscript{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.\textsuperscript{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, $\gamma$GT, 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 $\gamma$GT and ALT gave consistent results (AUC $\geq$ 0.75).

Alanine aminotransferase and $\gamma$GT are increased in HC dogs.\textsuperscript{5} Results of our study showed that undercontrolled HC dogs had significantly higher ALT and $\gamma$GT in comparison to well-controlled ones. In particular, values of ALT and $\gamma$GT 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{39}

The concentration of eACTH increases during trilostane treatment due to the loss of negative feedback regarding the cortisol concentration to the pituitary.\textsuperscript{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.\textsuperscript{17}

The ACTHst was not able to correctly identify the undercontrolled dogs according to previous investigations.\textsuperscript{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.\textsuperscript{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.\textsuperscript{7,8} However, all re-evaluations 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.\textsuperscript{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 re-evaluations 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

| Dog | Prepill cortisol (µg/dL) | Post-ACTH cortisol (µg/dL) | ALT (U/L) | $\gamma$GT (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: $\gamma$GT, gamma-glutamyl transferase; ALT, alanine aminotransferase; Hp, haptoglobin concentration; post-ACTH, post-ACTH administration cortisol concentration; prepill cortisol, before trilostane administration serum cortisol concentration.
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.

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OFF-LABEL ANTIMICROBIAL DECLARATION
Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION
The Ethical Committee of the University of Bologna Approved this protocol ID 607/2015.

HUMAN ETHICS APPROVAL DECLARATION
Authors declare human ethics approval was not needed for this study.

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