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Paper

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Pre-trilostane and three-hour post-trilostane cortisol to monitor trilostane therapy in dogs

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It is recommended that trilostane therapy of canine hyperadrenocorticism is monitored using an ACTH stimulation test, however this has never been validated. Three cortisol concentrations (pre-trilostane, 3-hour posttrilostane and 1-hour post-ACTH stimulation) were compared to a clinical score obtained from an owner questionnaire. There were 110 sets of 3 cortisol measurements and questionnaires obtained from 67 trilostane treated dogs. Questionnaire results were used to classify each dog as well or unwell. Well dogs were then categorised as having excellent, moderate or poor hyperadrenocorticism control, using thresholds produced by 14 independent veterinarians. Correlation co-efficients were used to compare the three cortisol concentrations to the owner score and the Kruskal Wallis and Mann-Whitney U tests were used to compare the three cortisol concentrations between categories of control. Cortisol cut-off values between significantly different categories were determined using ROC curves. Pre-trilostane and 3-hour post-trilostane cortisol were better correlated to the owner score and had cut-offs to differentiate between categories of control that had superior sensitivity and specificity results, than the post-ACTH cortisol. Iatrogenic hypoadrenocorticism was not detected in any unwell dog. This study shows that the pre-trilostane and 3-hour post-trilostane cortisol are potentially better monitoring methods than the ACTH stimulation test.

Trilostane is a competitive inhibitor of the enzyme 3-β-hydroxysteroid dehydrogenase, resulting in reduced synthesis of glucocorticoids and, to a lesser extent, mineralocorticoids (Potts and others 1978). Trilostane is licensed in many countries to treat pituitary and adrenal-dependent hyperadrenocorticism (HAC) (Vetoryl, Dechra). The manufacturer recommends a starting dose of 2 mg/kg once daily and frequent monitoring using a combination of history, physical examination and serum biochemical (including electrolytes) testing in combination with an adrenocorticotrophic hormone (ACTH) stimulation test started four-hour to six-hour post dosing (VMD 2016).

The ACTH stimulation test has been widely used and recommended as the monitoring method of choice for dogs receiving trilostane (Neiger and others 2002, Braddock and others 2003, Bell and others 2006, Vaughan and others 2008, Bonadio and others 2014); however, it has never been validated for this purpose. As trilostane is relatively short acting, there are concerns regarding the variation in results depending on the time since the last dose of trilostane (Bell and others 2006, Midence and others 2015) and whether results reflects clinical control (Wehner and others 2014). Recently, it has been suggested that as the nadir cortisol occurs three hours after trilostane administration, the ACTH stimulation test should be started two to four hours post dosing (Griebsch and others 2014).

Alternative methods of monitoring dogs receiving trilostane have been investigated with varying success. These methods include baseline cortisol (Cook and Bond 2010), endogenous ACTH and cortisol/ACTH ratio (Burkhardt and others 2013), haptoglobin (McGrotty and others 2005, Arteaga and others 2010) and the urine corticoid:creatinine ratio (UCCR) before and after trilostane (Galac and others 2009). In all of these studies, control was ultimately defined by the results of the post-ACTH stimulation cortisol and the owners' perception of clinical control is often not well described.

Tetracosactide (synthetic ACTH) can be expensive in some countries and has limited availability. Additionally, at high concentrations, it causes adrenal gland degeneration in rats (Burkhardt and others 2011). An effective monitoring tool for dogs receiving trilostane that does not require tetracosactide would be of considerable benefit.

The aim of this study was to develop an owner questionnaire to assess clinical control of dogs with HAC receiving trilostane. This measure of clinical control was then compared with the one-hour post-ACTH stimulation cortisol concentration (post-ACTH), the three-hour post-trilostane cortisol (also known as the baseline cortisol) (Cook and Bond 2010, Burkhardt and others 2013) and a novel monitoring method measuring cortisol concentration before trilostane was administered (pre-trilostane cortisol).

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Materials and methods

Case selection

Client-owned dogs were prospectively recruited from November 2013 to June 2014 from clinical practices throughout the UK including dogs referred to the Small Animal Hospital, University of Glasgow. External practices were recruited through a combination of personal contact with the authors and advertisement on a small animal internal medicine society website (SAMSoc 2013). Dogs were included if they had a pre-existing diagnosis of naturally occurring HAC that had been treated with a stable dose of commercially available trilostane (Vetoryl, Dechra) for at least 10 days. The results of testing to establish the original diagnosis and differentiation between adrenal and pituitary HAC, including endocrine testing and abdominal ultrasound results, were obtained retrospectively. This was through a combination of computer-based records and from telephone contact with the primary veterinary practice. The diagnosis of HAC was based on a combination of history, physical examination findings, haematology, biochemistry, urinalysis and endocrine testing (low-dose dexamethasone suppression test (LDDST), ACTH stimulation test or UCCR), which were compatible with HAC (Feldman 1983, Behrend and Kemppainen 2001, Behrend and others 2013). If the distinction between adrenal-dependent HAC and pituitary-dependent HAC was made, then it was recorded. Pituitary-dependent HAC was diagnosed if any of the following criteria were met; normal or high concentrations of endogenous ACTH, cortisol concentrations four-hour post-dexamethasone suppression below the lower limit of detection of the laboratory or less than 50 per cent baseline, pituitary enlargement on MRI or CT, or bilaterally symmetric normal-sized glands or adrenomegaly was considered consistent with (Barthez and others 1995, Feldman and others 1996, Van Liew and others 1997, Gould and others 2001, Melian and others 2010). In contrast, low or undetectable endogenous ACTH or an adrenal tumour with contralateral atrophy of the other adrenal gland was considered consistent with adrenal-dependent HAC (Barthez and others 1995, Feldman and others 1996, Van Liew and others 1997, Gould and others 2001, Melian and others 2010).

Throughout the study, the primary veterinarian remained in charge of case management. All changes in dose were made at their discretion and based on their own clinical assessments of the case.

Questionnaire

The primary veterinarian responsible for the care of each dog and the owner provided information regarding the signalment, date of diagnosis of HAC, trilostane dose (including frequency, duration, both in total and at the current dose), time of last trilostane administration, weight, abnormal physical examination findings, concurrent conditions and medications. Dogs were excluded if they were receiving medication or had a concurrent disease or physical examination findings that were likely to significantly impact on the cortisol results (e.g. pyrexia, glucocorticoid treatment (oral or topical) or severe illness such as malignant neoplasia) or if the owner had not given the dog its normal dose of trilostane the previous day. Dogs that had stable concurrent conditions such as hypothyroidism were not excluded. A questionnaire was developed from an ad hoc survey of practising veterinarians of the terms associated with HAC and their relative importance in assessing the clinical control of trilostane-treated dogs. The questionnaire was completed by the owner and was designed to both evaluate the owners' perception of the effectiveness of trilostane therapy and to highlight any dog that was unwell. Eight questions using a Likert scale response were used to assess thirst, urine volume, appetite, panting, exercise tolerance, coat quality, demeanour, gastrointestinal signs and the owner's overall impression of the HAC control. A ninth question (Q7) was directed at identifying other signs of progression of HAC, and for this the respondent was asked to tick all responses that applied to their pet. Weighted scores with a minimum total of 4 and a maximum total of 28 points available were retrospectively assigned to the answers that evaluated the effectiveness of HAC control by two of the authors (LM and IR). Answers that implied an increased severity of HAC signs resulted in a higher score assignment. No score was given to any answer that was a possible sign of illness; instead, the abbreviation PI (possible illness) was assigned each time an owner gave such a response. There were two answers that were equivocal (Q5a and Q9a) (i.e. the authors considered they could equally be consistent with illness or under control of HAC). These answers were given a score and PI was also assigned. A copy of the owner questionnaire, the scoring system and PI assignment are shown in online supplementary appendix 1. The information recorded on the questionnaire and from hospital records and contact with the primary veterinary practice were transferred to a database (Excel; Microsoft 2010). Dogs were excluded if they had incomplete or missing data.

The scores from the questionnaire were then retrospectively grouped into categories of clinical control that were defined by the following method. A group of 14 clinicians (6 junior clinical training scholars in small animal medicine and surgery with at least two years of relevant clinical experience, 4 ECVIM-CA residents in small animal internal medicine and 4 European specialists in veterinary internal medicine (DipECVIM-CA)) were individually asked to review answers from the completed owner questionnaire. A random number generator (Randomizer 2015) was used to group 30 questionnaires selected to represent a range of answers, into 10 groups of 3 each, and each veterinarian was given 3 groups of 3 questionnaires to assess (total 9 per person). The dog and owner details and the score from the questionnaire and/or PI assigned to each answer were not available to the clinicians.

The clinicians were first asked to categorise each dog as being well or unwell on the basis of the owners' answers. The clinicians' response was assessed, and a final category was reached by calculation of the mode for each of the 30 individual dogs. In both the dogs classified as well and those classified as unwell, the number of PI answers in each group was calculated. Using the results, the minimum number of PI answers required to classify a dog as unwell for the remainder of the study population was established.

The clinicians were then asked to group the well dogs into one of three categories of clinical control. The first was excellent control (defined as no dose increase likely required). The second two categories were degrees of under control; reasonable (small dose increase likely required) and poor control (large dose increase likely required). The clinicians' answers were assessed, and a final category was reached by calculation of the mode for each of the individual tests. The numerical scores that had been assigned to each dog by the authors were compared across the three categories of clinical control. Using the results, ranges of scores for each category of clinical control were established and then retrospectively applied to the remainder of the study population.

Blood sampling

Dogs were presented to the examining veterinary surgeon before receiving their trilostane dose. A blood sample was taken at the time of presentation (pre-trilostane). The trilostane dose was then administered, along with the dog's normal meal provided by the owner. Three hours after the trilostane had been given, an ACTH stimulation was performed by taking a blood sample (three-hour post-trilostane) and then administering 5 µg/kg of tetracosactide (Synacthen; Alliance) intravenously. A third blood sample was taken one hour after tetracosactide was administered (post-ACTH). The ACTH stimulation test was started at three hours because the cortisol nadir following trilostane administration occurs at this time in most dogs (Lehnert 2007, Griebsch and others 2014). The three blood samples were submitted to a single commercial veterinary laboratory (Veterinary Diagnostic Services, University of Glasgow) that takes part in a variety of quality

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assurance schemes including those run by Randox Laboratories and the European Society of Veterinary Endocrinology. Cortisol concentration was measured concurrently on all three samples using a competitive chemiluminescent enzyme immunoassay (Immulite 2000 cortisol; Siemens Healthcare, UK and Ireland) (Singh and others 1997) that has been validated for dogs and is widely used in laboratories throughout the world. Daily quality controls were performed in accordance with the manufacturer's instructions. Only results from samples ran on the same day in which paired standards had a coefficient of variation (CV per cent) less than 6 per cent were included in the analyses. The three cortisol results were transferred to the electronic database (Excel; Microsoft 2010) containing the dog details and owner responses from the questionnaire. The clinicians in charge of each case were provided with the three cortisol concentration results, but not the scores from the completed owner questionnaire or the categorisation (which were all applied retrospectively).

Unwell dogs

The dogs that were classified as unwell were further divided into two groups. The first included those with pre-trilostane or post-ACTH cortisol less than 40 nmol/l (which suggested they were unwell due to possible iatrogenic hypoadrenocorticism or another concurrent condition) and the second those with pre-trilostane or post-ACTH cortisol greater than 40 nmol/l (which suggested they were unwell secondary to another concurrent condition). The results from all these unwell dogs were not included in further analyses but assessed individually.

Statistical analyses

Statistical analyses were performed using commercially available software (IBM SPSS Statistics V.22, IBM; and Stata V.12.1, StataCorp). All continuous variables were assessed for normality using the Anderson-Darling method. The median values are reported for variables that were not normally distributed and the mean for variables that were normally distributed.

Pearson's correlation coefficient was calculated to determine the correlation between the three absolute cortisol results (pre-trilostane, three-hour post-trilostane and post-ACTH) to each other and the total owner score in the well dogs.

In the well dogs, the Kruskal-Wallis test was used to compare the cortisol concentrations between the three categories of clinical control, as defined by the results of the owner questionnaire. If a significant difference was found, a Mann-Whitney U test was performed between the three groups. A P value of <0.05 was considered significant.

If cortisol results were significantly different between groups, receiver operating characteristic (ROC) curves were constructed to evaluate the sensitivity and specificity of the pre-trilostane, three-hour post-trilostane and the post-ACTH stimulation cortisol at discriminating between the groups. ROC curves displayed sensitivity versus 1-specificity such that the AUC varied from 0.5 to 1.0, with higher values indicating increased discriminatory ability. Optimal cut-offs were determined such that the specificity was maximised while maintaining the sensitivity and therefore reducing the likelihood of unnecessary dose increases. Univariate and multiple stepwise mixed-effects linear regression analyses were used to assess any association between the three cortisol results (pre-trilostane, three-hour post-trilostane and post-ACTH) and the total owner score and the following factors: age, sex, bodyweight, total daily dose (mg/kg), dose frequency, duration at current dose (months), total duration of therapy (months), being well or unwell, type of HAC (adrenal or pituitary) and visit number. In the univariate analysis, a P value <0.2 was considered potentially significant and the factor was taken forward to multiple regression analysis. Variables were ordered by P value (smallest to largest) before sequential insertion into the multivariable models. Variables were retained in the models if $P<0.05$. Mixed-effects linear regression analysis was used to account for any potential effect of clustering within dog as some dogs were included in the database on more than one occasion.

Results

Dog signalment, method of diagnosis and visit information

In total, 110 tests were included from 67 individual dogs. A total of 37 tests were performed at the University of Glasgow and 73 tests were performed at external practices. In total, 94 tests were performed on dogs receiving once-daily trilostane and 16 on dogs receiving twice-daily trilostane. There were 6 female entire dogs, 38 female neutered dogs, 11 male entire and 12 male neutered dogs. At first presentation, the mean age was 11.19 years (sd ±2.53). The median weight was 11.85 kg (range 2.3–40 kg). The method of diagnosis was known in all dogs. The ACTH stimulation test was reported as the confirmatory test in 48/67 dogs and low-dose dexamethasone suppression in 19/67 dogs. There were 4 dogs diagnosed with adrenal-dependent and 23 dogs with pituitary-dependent HAC. In 40 dogs, the distinction was not made. To make the distinction, a combination of one or more of the following was used: abdominal ultrasound assessing adrenal size and symmetry in 17/27 dogs, LDDST in 9/27 dogs, endogenous ACTH in 4/27 dogs and MRI in 2/27 dogs. The median total daily dose of trilostane of all 110 tests was 3.47 mg/kg (range 0.67–16.51 mg/kg). The median duration of therapy before the first presentation was 4.5 months (range 0.33–36 months) and at the current dose was 2 months (range 0.33–36 months). The median of the total duration of therapy for all visits was 11.15 months (range 0.33–72 months).

There were 22 dogs that had more than one test performed consecutively; 9 dogs had 2 tests, 6 dogs had 3 tests performed, 6 dogs had 4 tests and 1 dog had 5 tests. Within this group, there were eight dogs that did not have a total daily dose or frequency change at any subsequent visit. There was one dog that had a frequency increase and one dog that had a frequency decrease, without a total daily dose change at a subsequent visit. A further five dogs had a total daily dose increase and three dogs had a dose decrease. There were three dogs that had both a frequency and dose increase at subsequent visits and one dog had a frequency and dose decrease.

Concurrent medication

In total, 15 of the 67 dogs received medication other than trilostane at one or more visits. These medications included meloxicam (Metacam; Boehringer) in three dogs and subcutaneous lente insulin injections (Caninsulin; Intervet) were reported in two dogs. Oral gabapentin (Neurontin; Pfizer), phenylpropanolamine (Propalin; Vetoquinol), benazepril (Fortekor; Elanco), clopidogrel (Plavix; Sanofi), tramadol (Tramadol; Actavis), levothyroxine (Thyforon; Dechra), ursodeoxycholic acid (Destolit; Norgine), amlodipine (Istin; Pfizer), a preparation containing green lipped mussel and glucosamine HCl (Yumove; Lintbells) and a preparation containing S-adenosyl-L-methionine, silybin, vitamin E and vitamin C (Samylin; Vetplus) were each prescribed in one dog.

Owner questionnaire results

From analysis of the clinicians' review of 30 of the owner questionnaires, if an owner answered three or more PI answers, then the dog was classified as being unwell. Using this protocol, 17 questionnaires from 11 dogs were classified unwell.

The total owners' scores for the 93 tests from 62 well dogs ranged from 5 to 25 (median=13). From analysis of the clinicians' assessments, the HAC control in the well dogs was further classified as excellent if they scored between 4 and 11, reasonable if they scored between 12 and 16 or poor control if they scored 17 or greater. Using this system, the control of HAC in 37 well dogs was classified as excellent, 33 as reasonable and 23 as poor. It was not possible for a test to score less than 4.

After analysis of the results from only the first visit of each of the 67 dogs, questionnaire answers indicated that 6 dogs were

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unwell and 61 dogs were well. Of these 61 well dogs, 23 were classified as having excellent control, 23 having reasonable control and 15 having poor control.

When the category allocation by the clinicians to the questionnaires was analysed, it emerged that all of the questionnaires were reviewed at least once by either an ECVIM-CA diplomate or a resident. When the results from only the ECVIM-CA diplomates or residents were compared with the results from all clinicians (i.e. including junior clinical training scholar), there was complete agreement in 28/30 (93.3 per cent) of the questionnaires. In the two questionnaires where there was disagreement, this was due to the inability to allocate the questionnaire into one category as the two ECVIM-CA diplomates/residents gave a different category of control.

Cortisol results: tests performed on well dogs

Absolute cortisol concentrations: all 93 tests

All three cortisol results were significantly correlated to the total owner score (pre-trilostane cortisol r=0.38, P<0.001; three-hour post-trilostane cortisol r=0.32, P=0.002; and post-ACTH stimulation cortisol r=0.27, P=0.01). All three cortisol results were also significantly correlated to each other (pre- and three-hour post-trilostane to post-ACTH r=0.56, P<0.001, r=0.68, P<0.001, respectively, and pre- to three-hour post-trilostane r=0.77, P<0.001).

When assessing differences between the individual groups, all three cortisol results were significantly lower in the dogs with excellent control compared with those with poor control. The pre-trilostane and three-hour post-trilostane cortisol were significantly lower in dogs with excellent compared with those with reasonable control. The post-ACTH cortisol was not significantly different between dogs with excellent compared with those with reasonable control. None of the three cortisol results was significantly different between dogs with reasonable control and those with poor control. Table 1 summarises the median and range of cortisol concentrations including relevant P values, between the clinical groups.

As there was no significant difference between the cortisol results of the dogs with reasonable control and poor control, and given both groups were defined as dogs with varying degrees of under control, test results from these groups were combined into one category (undercontrolled n=56). The pre-trilostane, three-hour post-trilostane and post-ACTH cortisol were all significantly lower in the dogs with excellent control compared with those that were undercontrolled (reasonable and poor control combined) (P<0.001, P<0.001 and P=0.027, respectively) (Fig 1). ROC curve analysis of pre-trilostane, three-hour post-trilostane and post-ACTH cortisols to distinguish those with excellent control from undercontrolled dogs had an AUC of 0.73, 0.73 and 0.64, respectively (Fig 2). Using a pre-trilostane cortisol of ≤138 nmol/l to distinguish those dogs that had excellent control from those that were undercontrolled gave a sensitivity of 55.4 per cent and a specificity of 86.5 per cent. Using three-hour post-trilostane cortisol of ≤62 nmol/l to distinguish those dogs that had excellent control from those that were undercontrolled gave a sensitivity of 58.9 per cent and a specificity of 81.1 per cent. Using post-ACTH stimulation cortisol of ≤130 nmol/l to distinguish those dogs that had excellent control from undercontrolled gave a sensitivity of 41.1 per cent and a specificity of 70.3 per cent.

Absolute cortisol concentrations: analysis of the first visit

The cortisol results from the first visit of each of the 61 individual dogs that were well were analysed. The pre-trilostane and three-hour post-trilostane cortisol were significantly correlated to the total owner score (r=0.344, P=0.007, and r=0.316, P=0.013, respectively). Unlike the complete data set, the post-ACTH stimulation cortisol was not significantly correlated to the total owner score (r=0.145, P=0.263).

The pre-trilostane and three-hour post-trilostane cortisol were significantly lower in dogs with excellent control compared with those with reasonable control and those with excellent control compared with those with poor control. The pre-trilostane and three-hour post-trilostane cortisol were not significantly different between those with reasonable control and those with poor control. Again, in contrast to the complete data set, the post-ACTH stimulation cortisol was not significantly different between any of the three groups. Table 2 summarises the median and range of cortisol concentrations, including relevant P values between the clinical groups.

When the dogs with reasonable and poor control were grouped together (undercontrolled group), the pre-trilostane and three-hour post-trilostane cortisol results were significantly lower in dogs that had excellent control compared with the undercontrolled group (P=0.020 and 0.033, respectively). Again, in contrast to the complete data set, the post-ACTH cortisol was not significantly different between dogs that had excellent control and the undercontrolled group. ROC curve analysis of the pre-trilostane and three-hour post-trilostane cortisol to distinguish those with excellent control from undercontrolled dogs had an AUC of 0.717 and 0.693, respectively. Using a pre-trilostane cortisol of ≤138 nmol/l to distinguish those dogs that had excellent control from those that were undercontrolled gave a sensitivity of 55.3 per cent and specificity of 87.0 per cent. Using three-hour post-trilostane cortisol of ≤62 nmol/l to distinguish those dogs that had excellent control from those that were undercontrolled gave a sensitivity of 52.6 per cent and a specificity of 73.9 per cent.

Cortisol results: tests performed on unwell dogs

There were 17 tests in 11 dogs categorised as unwell based on the results of the owner questionnaire. No owner answered 3a, 4d or 8a at any visit. The pre-trilostane was greater than 40 nmol/l in all dogs (range 46–508 nmol/l), as was the post-ACTH stimulation cortisol (range 52–375 nmol/l). It was therefore concluded that they were not unwell due to iatrogenic hypoadrenocorticism.

Low cortisol results

There were 31 results, all from well dogs, in which the three-hour post-trilostane cortisol was less than 40 nmol/l (range <7–39 nmol/l). In 2/31 tests, the pre-trilostane cortisol was concurrently low and in 6/31 tests the post-ACTH stimulation cortisol was concurrently low, which are described below.

TABLE 1: All 93 test results

	Excellent control (n=37)	Reasonable control (n=33)	Poor control (n=23)	P value (only significant results shown)
Pre-trilostane cortisol (nmol/l) median (range)	85 (22–323)*#	139 (54–480)*	155 (39–657)#	0.002* 0.001#
3-hour post-trilostane cortisol (nmol/l) median (range)	41 (<7–198)¥ †	74 (12–276)¥	85 (13–428) †	0.001¥ 0.003†
Post-adrenocorticotrophic hormone cortisol (nmol/l) median (range)	76 (7–353)Ø	103 (24–342)	121 (41–419)Ø	0.010Ø

The median and range of cortisol concentrations in the three categories of clinical control
*,#,¥,Ø,† depict cortisol results that are significantly different and associated P values are shown

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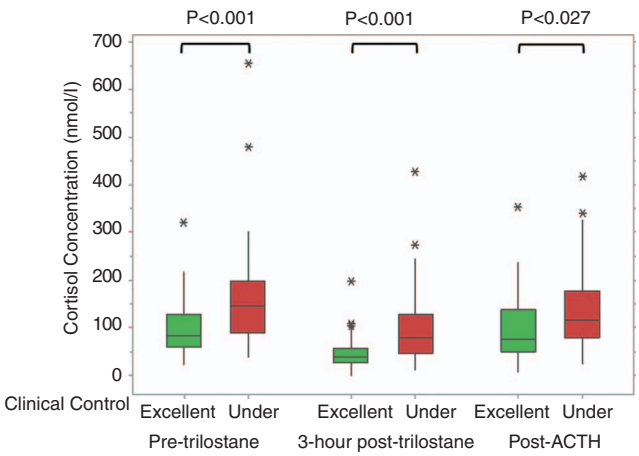

FIG 1: Box and whisker plots of pre-trilostane, three-hour post-trilostane and post-adrenocorticotrophic hormone (ACTH) cortisol concentrations divided into two groups of clinical control, an excellent (n=37) and an undercontrolled group (n=56) formed from the moderately and poorly controlled groups. The lower and upper boundaries of the box represent first and third quartiles of the data, respectively, with the line within the box representing the median. The whiskers represent the 5–95th percentile. * represents outliers. Significantly different results are indicated by connecting horizontal lines with the P values shown above

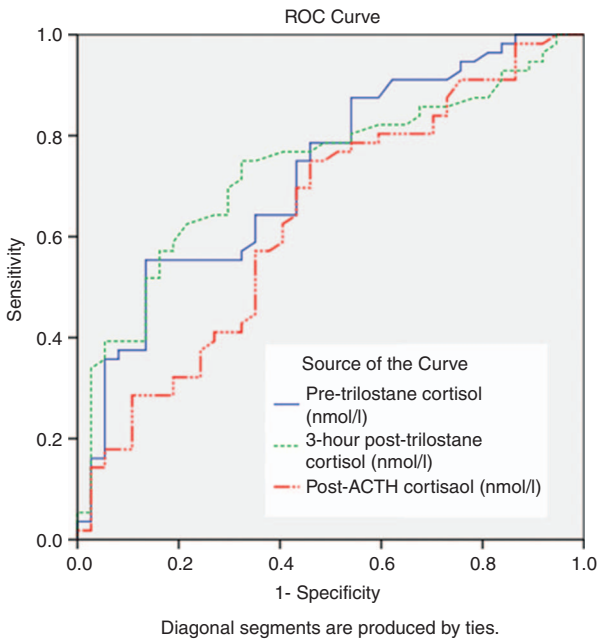

FIG 2: Receiver operating characteristic (ROC) curves assessing pre-trilostane, three-hour post-trilostane and post-adrenocorticotrophic hormone (ACTH) cortisol concentrations as a predictors of clinical control (excellent versus undercontrolled). The AUC for pre-trilostane cortisol was 0.73 (95% CIs) 0.62 to 0.83), three-hour post-trilostane cortisol was 0.73 (95% CI 0.62 to 0.84) and post-ACTH cortisol was 0.64 (95% CI 0.52 to 0.75)

There were eight post-ACTH stimulation cortisol concentrations that were less than 40 nmol/l (range 7–34 nmol/l). There were six pre-trilostane cortisol concentrations that were less than 40 nmol/l (range 22–39 nmol/l). Also, 5 of these 14 results were from the same dog, tested on three separate occasions. The other nine results were from different dogs. Therefore, in total there were 10 dogs that had a cortisol concentration less than 40 nmol/l at either or both time points. All dogs were classified as well based on the results of the owner questionnaire.

Follow-up information was obtained for all 10 dogs. All decisions regarding dose and frequency alterations were made by the primary veterinarian responsible for the care of each dog. There were eight dogs alive at the time of writing (median follow-up 584 days, range 525–663 days). In 4/8 dogs, the dose of trilostane was reduced after receiving the test results (three when post-ACTH cortisol and one when both the pre-trilostane and post-ACTH cortisol concentrations were less than 40 nmol/l). In two out of these four dogs, subsequent post-ACTH stimulation cortisol remained less than 40 nmol/l and the trilostane was stopped. In one of these dogs, the clinical signs of HAC returned within seven days and there was no record of subsequent clinical signs of HAC in the other. In the remaining 4/8 dogs, the dose of trilostane was initially not altered after receiving the cortisol results (2/4 when pre-trilostane and 2/4 when post-ACTH cortisol was less than 40 nmol/l). Subsequent follow-up revealed that in two of these four dogs the dose was reduced after the next visit, based on the results of repeat ACTH stimulation performed three to six hours after trilostane was administered. No record of clinical signs before or subsequent to this alteration was recorded and both dogs remained on the lower dose at the time of writing. In the remaining 2/4 dogs, the dose remained unaltered. In one of these two dogs, where only the pre-trilostane cortisol was less than 40 nmol/l, signs of acute gastroenteritis developed three months after the test. Hyponatraemia was detected at this stage; however, the potassium concentration and sodium:potassium ratio were normal. Trilostane was temporarily stopped but restarted at the same dose after successful treatment of the gastroenteritis. The other dog has remained clinically stable and both dogs receive the same dose of trilostane at the time of writing. In the dog with three separate tests with low cortisol results, two tests had both pre-trilostane and post-ACTH cortisol less than 40 nmol/l. One test had only pre-trilostane cortisol less than 40 nmol/l. The dose was reduced after each of the three test results. The first time only the pre-trilostane cortisol was low. Although the owner questionnaire indicated that the dog was well, after this dose reduction they reported the dog became brighter. The dose was reduced at both subsequent tests, in which both the pre-trilostane and post-trilostane were low. The owner reported on both occasions and at all subsequent visits that the dog was clinically normal and continues to receive the reduced dose for a further seven months.

Within this group of dogs, there were two dogs that had euthanasia performed during the follow-up period. One dog that had a post-ACTH cortisol less than 40 nmol/l but did not have a subsequent dose reduction had euthanasia performed 612 days after the result due to a pituitary macroadenoma. The other that had a pre-trilostane cortisol less than 40 nmol/l and had a subsequent dose reduction was euthanased 178 days after the result due to faecal and urinary incontinence. In both cases, iatrogenic hypoadrenocorticism was excluded immediately before euthanasia through a combination of haematology, biochemistry and endocrine testing.

Univariate and multivariable mixed-effects linear regression analysis

As the distinction between adrenal-dependent and pituitary-dependent HAC was only made in 27/67 dogs, this factor was not carried forward to multivariable analysis and only results with a $P<0.05$ from univariate analysis were considered significant.

In the univariate analysis of entire females, total duration of therapy and duration of current dose had significant ($P<0.2$) associations with pre-trilostane cortisol and were investigated in a multivariable model. In the final model, the results from the six female entire dogs compared with the remaining results were found to be associated with pre-trilostane cortisol ($P<0.05$). This model showed that pre-trilostane cortisol was a median of 93 nmol/l higher in entire female dogs compared with the rest of the population.

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TABLE 2: Test results from first visit only

	Excellent control (n=23)	Reasonable control (n=23)	Poor control (n=15)	p Value (only significant results shown)
Pre-trilostane cortisol (nmol/l) median (range)	83 (26–323)*#	139 (54–480)*	155 (39–657)#	0.013* 0.02#
3-hour post-trilostane cortisol (nmol/l) median (range)	38 (<7–198)¥ †	59 (20–276)¥	94 (25–428)†	0.032¥ 0.033†
Post-adrenocorticotrophic hormone cortisol (nmol/l) median (range)	102 (7–353)Ø	86 (24–196)	119 (41–419)Ø	0.1Ø

The median and range of cortisol concentrations in the three categories of clinical control
*,#,¥,Ø,† depict cortisol results that are significantly different and associated P values are shown

In the univariate analysis dose (mg/kg), duration of current dose, total duration of therapy and female entire had significant (P<0.2) associations with the three-hour post-trilostane cortisol and were investigated in a multivariable model. In the final model, total duration of therapy and female entire were found to be associated with the three-hour post-trilostane cortisol (P<0.05). This model showed that for every one-month increase in duration of therapy, the three-hour post-trilostane cortisol was a median of 1 nmol/l lower. In addition, the three-hour post-trilostane cortisol was a median of 46 nmol/l higher in the female entire dogs compared with the rest of the population.

In univariate analysis age, total dose (mg/kg), duration of current dose and total duration of therapy had significant (P<0.2) associations with the post-ACTH cortisol and were investigated in a multivariable model. In the final model, total duration of therapy and total dose mg/kg were found to be associated with post-ACTH cortisol (P<0.05). The model showed that for every one-month increase duration of therapy, the post-ACTH cortisol was a median of 2 nmol/l lower. In addition, for every 1 mg/kg increase in total dose, the post-ACTH cortisol was a median of 5 nmol/l higher.

In univariate analysis, duration of current dose, total duration of therapy, female entire and male entire had significant (P<0.2) associations with the total owner score in the results from well dogs and were investigated in a multivariable model. In the final model, the duration of current dose and female entire dogs compared with the remaining results were found to be associated with the total owner score (P<0.05). The model showed that for every increase in one month of therapy at the current dose, the total owner score decreased by a median of 0.14. In addition, the total owner score was a median of 5.1 higher in the results from female entire dogs compared with the rest of the population. Mixed-effects linear regression analysis demonstrated that there was no significant clustering within dog identity, and therefore, the inclusion of multiple visits from some dogs did not introduce a significant bias. Tables 3 and 4 summarise the results from univariate and multivariable mixed-effects linear regression analysis.

Discussion

The study showed that the pre-trilostane, three-hour post-trilostane and post-ACTH cortisol correlated with clinical control as defined by the veterinary assessment of owners' responses in a questionnaire. All three cortisol results were significantly lower in the dogs classed as having excellent control compared with those classed as being undercontrolled (reasonable and poor control combined). The pre-trilostane and three-hour post-trilostane cortisol were better than the post-ACTH stimulation cortisol at differentiating between the dogs with excellent control and those that were undercontrolled.

The aim of trilostane therapy should be to satisfactorily control clinical signs, without resulting in iatrogenic hypocortisolism. This study questions the existing recommendations that

TABLE 3: Univariate analysis investigating associations between factors and the pre-trilostane, the post-ACTH and the total owner score

	Pre-trilostane cortisol (nmol/l)n=110 dogs		3-hour post-trilostane cortisol (nmol/l)n=110 dogs		Post-adrenocorticotrophic hormone cortisol (nmol/l) n=110 dogs		Total owner score (well dogs only)n=93 dogs	
Factor	Coefficient	P value	Coefficient	P value	Coefficient	P value	Coefficient	P value
Age (years)	−1.43	0.71	−2.72	0.29	−4.55	0.15*	−0.18	0.34
Dose frequency	26.02	0.31	11.27	0.52	−13.65	0.53	−1.11	0.37
Well/unwell	1.80	0.94	9.46	0.58	16.55	0.43	N/A	N/A
Weight (kg)	−0.84	0.44	−0.68	0.36	−0.53	0.57	−0.07	0.24
Dose (mg/kg)	3.50	0.25	3.770	0.06*	4.92	0.05*	0.08	0.63
Duration at current dose (months)	−1.83	0.11*	−1.84	0.02*	−2.25	0.02*	−0.15	0.01*
Total duration of therapy (months)	−1.16	0.13*	−1.27	0.014*	−2.19	<0.01*	−0.09	0.02*
Type** (pituitary versus adrenal)	41.6	0.14	35.35	0.07	48.92	0.05	2.90	0.06
Sex (compared with female entire (n=8))								
Female neutered (n=59)	−82.95	0.02*	−41.84	0.07*	−26.00	0.39	−0.52	<0.01*
Male entire (n=22)	−91.11	0.01*	−46.18	0.07*	−31.25	0.34	−628	<0.01*
Male neutered (n=21)	−122.46	<0.01**	−71.45	0.01*	−31.27	0.34	−4.71	0.02*
Female entire versus all								
Female entire versus all	92.83	0.01*	48.87	0.04*			5.35	<0.01*
Visit number (compared with visit 1)								
2	−20.710	0.37	−11.28	0.47	23.93	0.22	−0.18	0.89
3	−12.273	0.67	−11.78	0.54	5.22	0.83	−0.27	0.86
4+	−25.744	0.46	14.47	0.55	37.40	0.21	−1.83	0.37

* indicates results with P<0.2 that were considered potentially significant
** indicates that because the distinction was only made in 27/67 dogs, with only 4/27 adrenal dependent, results were not carried forward to multivariable analysis and only results P<0.05 were considered significant for this factor
N/A, not applicable.

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TABLE 4: The final multivariable analysis model with factors retained (P<0.05)

	Pre-trilostane cortisol (nmol/l)n=110 dogs		3-hour post-trilostane cortisol (nmol/l)n=110 dogs			Post-adrenocorticotrophic hormone cortisol (nmol/l)n=110 dogs		Total owner score (well dogs only) n=93 dogs	
	Coefficient	p Value	Coefficient	R^2 (adj)	p Value	Coefficient	p Value	Coefficient	p Value
Dose (mg/kg)						4.78	0.04		
Duration at current dose (months)								−0.14	0.01
Total duration of therapy (months)			−1.22		0.02	−2.17	<0.01		
Female entire versus all	92.85	0.01	46.43		0.04			5.102	<0.01

post-ACTH stimulation cortisol should be used to both ensure adequate control and to detect dogs with oversuppression. In this study, two dogs with pre-trilostane cortisol less than 40 nmol/l and post-ACTH stimulation cortisol greater than 40 nmol/l were identified. One dog responded positively to trilostane withdrawal and a further case had signs consistent with hypoadrenocorticism three months later. The results also showed that many dogs with low post-ACTH cortisol did not rapidly develop hypoadrenocorticism. This is in agreement with a recent study that showed, in dogs that were well and had good control of their HAC, that post-ACTH stimulation cortisol measurements consistent with hypocortisolism at 3–6 hours did not persist and that measurements taken at 9–12 hours were significantly higher (Midence and others 2015). Continued trilostane therapy without altering the dose did not result in clinical evidence of hypoadrenocorticism in most of this group of dogs. A low concentration of pre-trilostane cortisol is potentially more significant than post-ACTH stimulation cortisol as this would be at least 12 hours (twice-daily dosing) or 24 hours (once-daily dosing) after the last dose of trilostane. An ACTH stimulation test performed at 24 hours could be used to assess the true adrenal reserve at this time (Bell and others 2006).

In previous studies that reported the use of post-ACTH stimulation cortisol as a monitoring tool for trilostane therapy, the clinical control was often poorly defined. There are no studies demonstrating good correlation between a systematic evaluation of owners' observations of clinical control and the post-ACTH stimulation cortisol. There is also a lack of consensus regarding the target post-ACTH stimulation cortisol concentration in trilostane-treated dogs with values varying from 15 to 250 nmol/l used to define good clinical control (Neiger and others 2002, Ruckstuhl and others 2002, Braddock and others 2003, Wenger and others 2004, Barker and others 2005, Galac and others 2010, Ramsey 2010).

In this study, both the pre-trilostane and three-hour post-trilostane cortisol were better able to discriminate between dogs that had excellent control from those that were undercontrolled. Measurement of either or both is potentially easier and less expensive than an ACTH stimulation test. Of the two results, the pre-trilostane cortisol was slightly better than the three-hour post-trilostane cortisol and had the added benefit of being more useful at signalling potential oversuppression. Using the three-hour post-trilostane cortisol (or baseline cortisol) has previously been evaluated as a monitoring tool. Similar to the authors' findings, it was difficult for the authors to recommend as a stand-alone test as it indicated oversuppression in too many cases (Burkhardt and others 2013). Further research into using a combination of both the pre-trilostane and three-hour post-trilostane may be useful.

In this study, the authors suggest a potential target range for pre-trilostane cortisol of greater than 40 and less than 138 nmol/l. A cut-off of 138 nmol/l was chosen to maximise the specificity of results below this limit, indicating excellent control. The lower limit of 40 nmol/l was chosen as cortisol concentrations above this generally are considered to exclude hypoadrenocorticism in trilostane-treated dogs (Cook and Bond 2010, Ramsey 2010, Burkhardt and others 2013). Further studies, particularly including more dogs with trilostane-induced hypoadrenocorticism, could alter these ranges, and it is debatable as to whether specificity or sensitivity should be maximised.

All cortisol assays in this study were measured in a single lab using a validated and widely used chemiluminescent enzyme immunoassay technique. However, there are clinically significant differences between alternative assays and even laboratories using the same techniques (Russell and others 2007, Behrend and others 2013). It is difficult to extrapolate results and cut-off values from this study to other laboratories, particularly those using an alternative method of measuring cortisol.

There were several factors identified as having a potential influence over the pre-trilostane, three-hour post-trilostane and post-ACTH cortisol concentrations as well as the total owner score. Female entire dogs had a significantly higher pre-trilostane and three-hour post-trilostane cortisol concentrations and total owner scores. It has been shown that healthy entire male dogs have lower post-ACTH simulation cortisol (Frank and others 2003) but others have not found an influence of sex and neuter status on cortisol concentrations (Reimers and others 1990). The number of results from female entire dogs was small, which could have impacted on the results.

It was also interesting to note that as the trilostane dose increased this was associated with a small increase in post-ACTH cortisol concentrations. This further demonstrates the difficulty in predicting or interpreting the results of post-ACTH cortisol as a monitoring tool. Alternatively, it could reflect that dogs requiring a higher dose are more refractory to therapy. In addition, the longer the dogs were receiving the current dose the lower the total owner score. This suggests that either there is a gradual improvement in signs or there is a degree of acceptance by the owners of their dog's current clinical condition as being the 'new normal' for that dog. As the total duration of therapy increased, both the three-hour post-trilostane and the post-ACTH stimulation cortisol decreased, suggesting a time-dependent suppression of both (as this was not associated with increased total dose).

The dogs were brought to and kept in a hospital for the duration of the test (approximately four hours), and it is possible that these environmental stresses could alter cortisol results (e.g. a car journey might increase the pre-trilostane cortisol in a dog that is distressed by travelling). It has previously been shown that an examination and hospitalisation can increase urinary cortisol to creatinine ratios (van Vonderen and others 1998); however, the testing environment (including whether hospitalised or at home) did not markedly affect the results of ACTH stimulation testing in normal dogs (Vial and others 1979). Additionally, as the dogs were fed in hospital, it is possible that this could influence trilostane absorption and therefore efficacy as it is known that stress can result in a delay in gastric emptying (Enck and Holtmann 1992). It remains unknown as to what effect this factor could have on absorption of the drug and therefore on the results of the pre-trilostane and post-ACTH stimulation cortisol. Further investigation is warranted; however, this factor would have little or no influence on the pre-trilostane cortisol concentration. Particular care was taken to ensure that wherever possible the dog's normal food was provided by the owner during the test.

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One limitation of this study is that the majority of dogs were not differentiated into adrenal-dependent or pituitary-dependent HAC. The authors chose to include all cases of HAC as this reflects the general population of dogs treated with trilostane.

A second limitation is that the number of dogs receiving twice-daily trilostane was small. The dose and frequency of administration of trilostane was selected by the dogs' primary veterinary surgeon. However, dose frequency was not identified in univariate analysis as having a significant impact on any of the three cortisol results or the total owner score. In dogs receiving trilostane once daily, the duration of suppression of cortisol varies between 2 and 13 hours after trilostane (Bell and others 2006). In addition, there is no significant difference between the cortisol 12 and 24 hours after trilostane administration (Griebsch and others 2014). It is therefore to be expected that the pre-trilostane cortisol concentration was similar in dogs treated with once-daily or twice-daily trilostane. However, larger case numbers are required to draw definitive conclusions.

In this study, the definition of the dog's clinical control was based on the results of a standardised clinical history obtained by means of owner questionnaire. The results of the questionnaire were assessed and categorised according to a consensus of clinicians who were blinded to the actual cases. The clinicians had varying levels of experience and expertise, and this could have impacted on their ability to judge and categorise the answers given. However, as veterinarian assessing dogs with HAC vary in their experience, the inclusion of junior clinician training scholars into the assessing group of clinicians better reflects this population. It could be suggested that the questionnaire be used alone to make decisions regarding dose adjustment. However, the questionnaire does not provide definitive evidence of trilostane overdose until clinical signs develop, whereas it is speculated that cortisol measurements may provide earlier warning of an overdose. In addition, owners may vary in their abilities and could underestimate or overestimate the clinical signs at home and/or fail to recognise the signs of hypoadrenocorticism. Furthermore, veterinarians vary in their experience of HAC and the decisions made by specialists working in a referral hospital using a questionnaire may not be so easily extrapolated to a busy first opinion mixed practice.

The questionnaire was developed from an ad hoc survey of practising veterinarians. Ideally, this would have been tested through a validation process, following established psychometric approaches (Juniper and others 1996, Wiseman-Orr and others 2006). The process of development and implementation of this questionnaire occurred during a time when tetracosactide (Synacthen; Alliance) supply suddenly declined in the UK. Therefore, it was not possible to test and validate this questionnaire in the time frame required to commence this study. Future studies including development of a questionnaire following established psychometric approaches could be considered. It should be noted that the results obtained in this study are similar to those obtained with other questionnaires in other languages, suggesting a common theme (Wehner and others 2013).

The majority of the dogs did not have biochemistry, electrolytes and haematology performed at the time of sampling, despite the manufacturer's datasheet recommendations (Vetoryl, Dechra (VMD 2016)). It is possible that some dogs had developed subclinical hyperkalaemia, which may have indicated the development of iatrogenic hypoaldosteronism in the absence of hypocortisolism. However, dogs can become mildly hyperkalaemic (subclinical) after initiation of trilostane therapy, and there is no correlation between aldosterone concentration and potassium (Wenger and others 2004, Reid and others 2014). Additionally, there was no difference between potassium concentrations in dogs with post-ACTH stimulation cortisol less, or greater, than 41 nmol/l (Ruckstuhl and others 2002). Although trilostane can result in suppression of post-ACTH stimulation aldosterone (Reid and others 2014), the clinical relevance and value of frequent monitoring of electrolytes in well dogs receiving trilostane remain unclear as both sodium and potassium appear to be both in insensitive and non-specific markers of decreased aldosterone reserve in trilostane-treated dogs. In addition, the value of repeated haematology and biochemistry remains questionable as many dogs treated with trilostane had persistent haematological alterations (lymphopenia/monocytosis) and increased alkaline phosphatase, despite adequate clinical control (Ruckstuhl and others 2002, Arteaga and others 2010). Further studies are needed to assess the value of biochemical profiles when pre-trilostane or post-ACTH stimulation cortisol concentrations are less than 40 nmol/l.

There were 17 questionnaires from 11 dogs with results that suggested the dog was unwell. None had a cortisol concentration less than 40 nmol/l at either point, and it was assumed that they were unwell for another reason. These dogs were excluded from further analysis because the dogs' cortisol may have been affected by another unrelated concurrent illness and therefore may not be reliable for the assessment of clinical control (Kaplan and others 1995).

Initially, the dogs were categorised into those with reasonable or poor control. The initial aim was to ascertain whether the cortisol results would be sensitive enough to differentiate between degrees of under control. This could help clinically to determine increments of dose changes. However, as none of the three cortisol results was significantly different between these two subcategories of clinical control and both described degrees of ineffective control, the authors elected to combine both into one 'undercontrolled' category.

In conclusion, the results of this study show that the pre-trilostane and three-hour post-trilostane cortisol concentrations were better than the post-ACTH cortisol concentrations at discriminating between dogs with excellent control and those that were undercontrolled. No cortisol result correlated well enough with the clinical score to be used as stand-alone monitoring test, and it may be that the only effective monitoring tool would be one based on clinical signs. However, the pre-trilostane cortisol was the objective measurement that had the most potential to balance safety with effective therapy. Further studies fully assessing the repeatability of the cortisol results and the inclusion of dogs that are unwell due to iatrogenic hypoadrenocorticism are needed before this novel method can be used as an adjunctive monitoring tool.

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