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AUTHORS: E.J. Knowles, M. C. Moreton-Clack, S. Shaw, P. A. Harris, J. Elliott, N. J. Menzies-Gow

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MR. EDWARD JOHN KNOWLES (Orcid ID : 0000-0001-9440-6105)

DR. NICOLA J MENZIES-GOW (Orcid ID : 0000-0002-3803-8069)

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Plasma adrenocorticotrophic hormone (ACTH) concentrations in ponies measured by two different assays suggests seasonal cross reactivity or interference

E. J. Knowles^{1,2}, M. C. Moreton-Clack², S. Shaw³, P. A. Harris⁴, J. Elliott¹ and N. J. Menzies-Gow¹

¹ The Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, Hertfordshire, AL9 7TA, UK;

² The Bell Equine Veterinary Clinic, Butchers Lane, Mereworth, Kent, ME18 5GS, UK;

³ Tosoh Bioscience Ltd, Edward St, Redditch, Worcestershire, B97 6HA, UK;

⁴ Equine Studies Group, WALTHAM Centre for Pet Nutrition, Waltham on the Wolds, Leicestershire, UK.

***Corresponding author email:** e.j.knowles@gmail.com

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Summary

Background: Analysis of plasma adrenocorticotrophic hormone concentration [ACTH] aids diagnosis of pituitary *pars intermedia* dysfunction (PPID). Comparisons of validated chemiluminescent-immunoassay (CI) and immunofluorescent (IF) assays are limited.

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Objectives: To compare the results of [ACTH] analysis by CI and IF methods of samples collected in autumn and spring and assess cross-reactivity.

Study design: Method comparison.

Methods: Plasma from non-laminitic ponies was analysed concurrently using the IF and CI methods in autumn and the following spring. Diagnostic thresholds for the IF method were derived using ROC curves and Youden indices to correspond with CI thresholds. Assay specificity was assessed using commercially available ACTH fragments and degradation products of endogenous ACTH.

Results: CI and IF methods yielded different results ($p < 0.001$); mean differences (CI-IF), (95% confidence intervals): Autumn ($n = 99$) 38.6 (30.6-46.5) pg/ml, Spring ($n = 88$) 5.1 (3.9-6.3) pg/ml.

The association between CI and IF results differed in autumn and spring, consistent with seasonally dependent cross-reactivity or interference. Good ($\kappa = 0.66-0.74$) agreement was obtained for binary interpretation in spring between IF and CI using thresholds of >24 pg/ml and >29 pg/ml respectively and in autumn between IF and CI using thresholds of >27 pg/ml and >47 pg/ml respectively or >33 pg/ml and >77 pg/ml respectively. Of 88 ponies with both spring and autumn samples, 56 (64%) exceeded a published autumn CI threshold (>47 pg/ml), of which 39 (70%) were below the equivalent threshold (<29 pg/ml) the following spring without treatment. The CI assay showed substantial apparent increases in [ACTH] following addition of high concentrations of CLIP (ACTH 18-39). Degradation of ACTH during storage affected the assays differently.

Main limitations: Limited numbers of PPID cases were included. Immunoreactivity of commercially available peptides may differ from their endogenous equivalents.

Conclusions: The methods yielded different absolute values but the agreement for binary classification was good. An altered pituitary secretome in autumn that affects apparent [ACTH] values seems likely.

Introduction

Equine pituitary *pars intermedia* dysfunction (PPID) is a common disease of older horses and ponies [1]. Plasma concentrations of the pro-opiomelanocortin (POMC) derived peptide adrenocorticotrophic hormone [ACTH] are often measured to aid diagnosis [1]. Seasonally adjusted reference ranges are recommended due to a physiological increase in pituitary hormone output in autumn [1]. In a large study in the United Kingdom, basal plasma [ACTH] >29 pg/ml in non-autumn months and >47 pg/ml in the autumn, measured using a chemiluminescent immunoassay (CI) [2], were consistent with a diagnosis of PPID [3]. Alternative diagnostic thresholds using the same method have been derived from other populations, including >77 pg/ml in autumn months in Australia [4]. A 'grey zone' of 19-40 pg/ml in non-autumn months has been proposed, within which results may be considered equivocal and re-testing at a later date or alternative tests may be indicated [5]. Recent discussion suggests the thresholds for diagnosis and the extent of 'grey/equivocal zones' warrant further review[6].

In addition to the CI method, an automated ACTH immunofluorescence assay (IF) has recently been validated in the horse [7]. A previous study [7] to compare [ACTH] measured by the CI and IF methods concluded that correlation between the methods was good but with significant bias. Seasonal effects were not analysed and the study included only a small number (n = 4) of samples with very high measured ACTH concentrations (>100 pg/ml by the CI method).

ACTH is a 39 amino-acid fragment of pro-opiomelanocortin (POMC) [1]. The manufacturers of the CI immunoassay report 13-15% cross reactivity with the C-terminal fragment of ACTH (ACTH 18-39) [8] known as corticotropin-like intermediate lobe peptide (CLIP). It is unknown whether this cross reactivity is clinically relevant *in vivo* in equine samples. No CI cross reactivity is reported to α -MSH (ACTH 1-17), or β -cell troponin (ACTH 22-39). No cross reactivity is reported by the manufacturers of the IF assay.

The study had 3 aims, namely

- 1) In a cohort of ponies with no known history of laminitis, to compare the results of [ACTH] analysis by CI and IF methods in samples collected in the spring and autumn and to determine whether the two different methods provide equivalent categorical interpretation (PPID positive or negative at each time) when published diagnostic thresholds are used.
- 2) In the same cohort, to determine whether the results of [ACTH] analysis were within a published (seasonally adjusted) reference range for the CI analyser [3] when tested in the same animals in the spring and the autumn.
- 3) To assess the cross-reactivity of both assays using synthetic human ACTH fragments and by assessing the effect of delayed plasma analysis.

Methods

Ethical approval for the study was obtained from the Royal Veterinary College Ethics and Welfare Committee and the study was conducted under a UK Home Office licence (PPL 70/8195).

Population study

Subjects

A subset of ponies from a larger on-going cohort study were included. Briefly, pony owners were recruited through internet searches and equestrian societies to obtain contact details for owners of groups of ponies kept within the county of Kent or within a 25-mile radius of the postcode ME18 5GS. Pony owners were invited to participate in a cohort study investigating laminitis risk factors. Owners that were willing to participate were included if they expected to have at least 5 eligible ponies. Ponies were eligible for inclusion if they were ≤ 148 cm (149 cm with shoes), ≥ 5 years old and had no known history of laminitis. Ponies that were known to be pregnant, were receiving treatment for PPID or were clinically unwell at the time of examination were excluded from the cohort. The first 100 ponies to be recruited for the main cohort were eligible for inclusion in the study.

Data collection

Each pony was visited during the autumn (September or October 2015) and then again in the following spring (March or April 2016). On each occasion, blood was collected for analysis of [ACTH]. Clinical signs of PPID noted by the owner or evident on veterinary examination were recorded in spring 2016. Specifically, the presence of hypertrichosis, a 'pot belly' or bulging supraorbital fat pads were assessed subjectively on veterinary examination. Owners were asked if each pony had suffered from laminitis or shown any of the following signs: lethargy or lack of energy, muscle wastage, supraorbital fat (fat bulging around the eyes), drinking or urinating excessively, long abnormal coat or delayed winter coat shedding, repeated infections. In spring 2016, the veterinary surgeon performing the examinations and the pony owners were blinded to the results of the ACTH analysis from autumn 2015 other than in one case that was unblinded for welfare reasons.

Blood collection and ACTH analysis

Blood was collected from the jugular vein into EDTA plastic vacutainers^a. Whole blood was immediately chilled on ice packs to 4-10°C until it was separated by centrifugation (10 min at 2000 x g) the same day. Following centrifugation, plasma was aliquoted and stored at -20°C in the short term (up to 6 days) prior to longer term storage at -80°C. Samples were anonymised prior to analysis within 3-6 months.

Plasma was thawed on the day of analysis. Samples were separated into 2 aliquots and analysed concurrently using two different automated analysers, a chemiluminescent immunoassay (ACTH Immulite 1000^b (CI) and an immunofluorescent immunoassay (ST AIA ACTH^c) (IF). The CI assay is a solid phase, 2-site sequential chemiluminescent immunometric assay that uses mouse monoclonal and rabbit polyclonal anti-human ACTH. The IF assay is a 2-site immunoenzymometric assay which uses 2 types of polyclonal goat anti-human ACTH antibodies [7]. Analysis and quality controls were performed according to manufacturers' instructions. Any samples with significant haemolysis were excluded from analysis.

Cross reactivity/interference

Cross reactivity to or interference by commercially available ACTH fragments was assessed using two pools of residual plasma from animals in the main cohort (samples taken in autumn 2016 and spring 2017). The plasma samples had been thawed and analysed previously then stored at -20°C for up to 6 months. The peptides α -MSH (ACTH 1-17)^d, CLIP (ACTH 18-39)^d and β -cell troponin (ACTH 22-39)^d were obtained from a commercial supplier and had identical amino acid sequences to the human ACTH fragments. Each peptide was reconstituted with distilled water in borosilicate glass tubes^d and serially diluted to produce working concentrations. Small volumes of synthetic peptide were added (spiked) to the pools of spring and autumn plasma to achieve peptide concentrations from 0-32000 pg/ml. Each spiked sample was analysed concurrently using the IF and CI methods.

Cross reactivity/interference from the natural degradation products of endogenous ACTH was assessed by analysing aliquots of plasma that had been stored at room temperature for 24, 48, 72 and 96 hours to allow partial degradation of ACTH. Residual plasma from 10 clinical cases assessed for PPID by Bell Equine Veterinary Clinic were used, sample handling and temporary storage complied with clinical recommendations [13]. An aliquot of each sample was analysed concurrently using the IF and CI analysers on day 0 (for diagnostic purposes) and every 24 hours (days 1-4) until 96 hours had elapsed.

Data Analysis

Clinical signs were reported as descriptive data. When the number of animals in each group was sufficiently large, associations between clinical signs and [ACTH] by each method were tested using Mann-Whitney U tests. Associations were considered significant if $p < 0.05$ (a Bonferroni correction was applied for multiple comparisons).

To determine whether there was an absolute difference between the results obtained using the CI and IF analysers the sample means for each method (from concurrent analysis) were compared by

paired sample *t* tests in the spring and autumn separately. The relationship between plasma ACTH concentrations as measured by the different methods were examined by scatter plots of the autumn and spring results. Linear regression was performed to examine the relationship between results generated using the two methods. Regression coefficients for spring and autumn were compared with an F test. The normality of the distributions of the differences between paired values and the residuals of linear regression were assessed using histograms. Percentage difference Bland-Altman plots [9] were created to show the limits of agreement between the methods in spring and autumn.

To determine whether equivalent categorical agreement could be obtained using the different methods, spring and autumn thresholds were derived for the IF analyser to correspond to the published thresholds of >29 pg/ml and >47 pg/ml using the CI in the spring and autumn respectively [3]. Thresholds were also derived to correspond to an alternative autumn CI threshold derived by McGowan *et al.* (>77 pg/ml) [4] and to the upper and lower limits for equivocal results to correspond with the suggested 'grey zone' for the CI analyser (19-40 pg/ml) in non-autumnal months [5].

Thresholds for the IF were derived by generating ROC curves [10] for each CI threshold and calculating the whole number threshold with the highest Youden index [11]. To describe the agreement between the IF and CI methods if these thresholds were applied to IF results, Kappa statistics were calculated for a binary interpretation of [ACTH]. Weighted kappa (with linear weights for the disagreements) was calculated for the ordinal interpretation of results when the 'grey zone' for equivocal results was included [12]. Kappa values of >0.81 were considered to indicate very good agreement, 0.61-0.8 good, 0.41-0.6 moderate, 0.21-0.4 fair and <0.2 as poor [12].

To determine whether the result of plasma ACTH analysis were within a published reference range [3] when tested in the same animals in the spring and the autumn, only CI results were analysed.

Data from ponies that were unavailable in spring 2016 were excluded. Samples with [ACTH] >47 pg/ml in autumn and >29 pg/ml in spring were considered positive for PPID.

Cross reactivity to synthetic peptides was not analysed statistically. For degradation of endogenous ACTH the percentage recovery of ACTH for each assay for days 1-4 was calculated as the percentage of the original (day 0) [ACTH] value for that assay. A mixed effects model was used to assess differences in [ACTH] recovery between assays. The dependent variable was [ACTH] percentage. Day, assay and their interaction were entered as fixed effects with day within subject as random effects (with AR1 covariance) using statistical software^e. Recovery for each day was compared and differences were considered significant if $p \leq 0.05$, a Bonferroni correction was applied.

All analyses were performed using statistical and graphical software^{e,f,g}.

Results

Population study

Blood samples were included from 99 ponies in autumn 2015 (one pony was excluded for behavioural reasons). There were 59 geldings and 40 mares with a mean age of 12.8 years (range 5-27 years). Breed information was available for 85 ponies: 32 were described as Welsh or Welsh X, 13 as Shetland, miniature Shetland or Shetland X (excluding Shetland x Welsh), 12 as Cob or Cob X, 6 as New Forest, 4 as Highland and 17 as other pony breeds or crosses. Eleven ponies were not used for any ridden exercise whereas all others had some ridden exercise.

In spring 2016, 7 ponies had moved yards and were unavailable for re-sampling, one pony had developed laminitis and was therefore excluded from the cohort, a blood sample could not be obtained from one pony for behavioural reasons and two blood samples showed marked haemolysis and were excluded from analysis. Therefore, samples from 88 ponies were available for analysis in spring 2016. Complete data, including owner and veterinary assessment of clinical signs, were available for 86/88 ponies.

Box and whisker plots of [ACTH] as measured by each method and during the autumn and spring are shown in Figure 1. The distribution of the data was right skewed. Median, mean, (range) of the samples for each method were: autumn CI: 54.8, 74.0, (20.8 -328) pg/ml, spring CI: 20.5, 23.6, (11.2-142.1) pg/ml, autumn IF: 27.6, 35.4 (10-142) pg/ml, spring IF: 16, 18.5 (4.3-59.3) pg/ml.

There was a significant difference between the results obtained using the two methods in both autumn and spring. The mean differences (CI – IF), (95% confidence intervals and p values) were: autumn 38.6 (30.6-46.5 pg/ml $p < 0.001$), spring 5.1 pg/ml (3.9-6.3 pg/ml, $p < 0.001$). Bland-Altman analysis revealed a mean bias (limits of agreement) of 64.9% (17.1%-112.7%) in autumn and 28.8% (-28%-85.6%) in spring (Supplementary Item 1).

The associations between the values obtained using each assay are shown in Figure 2. Panel A shows all data. Some autumn samples had very high ACTH values, therefore to show the association between the analysers within a similar range of values panel B shows the associations between methods when [ACTH] values > 100 pg/ml using the CI assay were excluded (16 autumn samples were excluded). Linear regression of all data indicated that in the autumn: IF (pg/ml) = $0.34 \times \text{CI} + 10.2$ ($r^2 = 0.38$) and in the spring: IF (pg/ml) = $0.86 \times \text{CI} - 1.8$ ($r^2 = 0.68$). The relationship between the CI and IF assays differed significantly ($p < 0.001$) between spring and autumn.

Receiver operating characteristic (ROC) curve and Youden index calculations indicated that an IF spring diagnostic threshold of > 24 pg/ml produced the greatest agreement with the previously recommended non-autumn CI threshold of > 29 pg/ml. In the autumn, IF thresholds of > 27 pg/ml and > 33 pg/ml gave the best agreement with the previously recommended autumn CI thresholds of > 47 pg/ml and > 77 pg/ml respectively. Using these IF thresholds, the agreement in binary classification was good. An IF 'grey zone' of 18-24 pg/ml for equivocal values gave greatest agreement with the non-autumn CI 'grey zone' (19-40 pg/ml). Agreement (weighted kappa) with an ordinal

interpretation (normal, grey zone or abnormal) was moderate. Thresholds and Kappa statistics are shown in Table 1.

Seasonal variation in ACTH concentrations

Both autumn and spring samples were available for analysis from 88 ponies. The results of classification of each pony as PPID positive or negative in the autumn and the following spring based on published thresholds of 47 pg/ml and 29 pg/ml [3] using the CI assay are shown in Table 2. In autumn 2015 56/88 (64%) were classified as positive ([ACTH]> 47 pg/ml). In spring 2016 20/88 (23%) were classified as positive ([ACTH]> 29 pg/ml). Of the 56 ponies classified as positive in the autumn, 39 (70%) were within the reference range the following spring. None of the ponies received pharmaceutical treatment for PPID during this period.

Clinical signs of PPID

Of the 88 samples used for analysis in spring 2016, complete clinical data were available for 86. Veterinary examination data was missing for one pony and owner information was missing from one pony. On veterinary examination, 3 (3%) ponies were considered to have hypertrichosis, 10 (12%) had bulging supraorbital fat pads and 18 (21%) were considered to have a pot belly. Owners reported a long abnormal coat or delayed winter coat shedding in two ponies (2%) (one of which was considered to have hypertrichosis on veterinary examination). Both lethargy and excessive drinking and urinating were reported in one pony (1%), muscle wastage was reported in one additional pony (1%). No owners considered their ponies to have recurrent infections or bulging supra-orbital fat pads. No signs consistent with PPID were present on veterinary examination or reported by owners in 59 (69%) of ponies. Insufficient clinical signs were reported by owners for statistical analysis statistical analysis was therefore only performed on signs that were noted on veterinary examination. Of the veterinary apparent signs, hypertrichosis was associated with [ACTH]

for both methods during both seasons ($p < 0.01$ - $p = 0.012$), there were no significant associations between bulging supra-orbital fat pads or a pot belly and [ACTH].

Cross reactivity study

The results of addition of synthetic ACTH fragments are shown in Figure 3 and Supplementary Item 2. One sample did not yield a result due to a sampling error (aspiration of a bubble). In summary, addition of CLIP at high concentrations substantially increased apparent [ACTH] using the CI assay but had no effect on the IF assay, although the percentage cross reactivity was low (1-2% at [CLIP] \geq 2000 pg/ml for the CI assay). At high concentrations α -MSH reduced apparent [ACTH] using the IF assay but had no apparent effect on the CI assay. High concentrations of β -cell troponin also reduced apparent [ACTH] using the IF assay and the CI assay at the very highest concentration (50 000 pg/ml) only.

The results of analysis of endogenous ACTH during storage for up to 96 hours at room temperature are shown in Figure 4. There was insufficient remaining sample on day 4 for analysis of 3 samples. Mixed effect model analysis revealed that recovery of [ACTH] was significantly higher for the CI assay than the IF assays on days 3 and 4 ($p = 0.032$ and < 0.01) consistent with endogenous ACTH degradation products cross reacting with the CI assay or interfering with the IF assay.

Discussion

The results of the current study are consistent with those of Irvine *et al.* [7] in which the results obtained with the IF assay were proportional to, but lower than, those obtained using the CI assay. In the current study, a different relationship was found between [ACTH] results generated by the two methods when measuring concentrations in samples collected from the same animals in the autumn compared with the spring. A probable explanation for this finding is cross reactivity to or interference by a substance present in equine plasma at greater concentrations in the autumn than

the spring, such as other POMC derived peptides. Whilst both the CI and IF assays have previously undergone similar validation procedures in the horse [2,6], studies in the horse have not fully investigated cross reactivity of either assay to other POMC derived peptides, nor compared assay results with gold standard techniques such as liquid chromatography-mass spectrometry (LC-MS).

The occurrence of cross reactivity or interference was firstly investigated by the addition of commercially available synthetic ACTH fragments to equine plasma samples. Cross-reactivity or interference was demonstrated with both assays. The most marked effect was of CLIP on the CI assay; however, for both assays these effects only occurred at concentrations of the three ACTH fragments that are likely to be supraphysiological. The calculated cross reactivity of 1-2% to CLIP in equine plasma was surprisingly low given the manufacturer's report of 13-15% cross reactivity in human plasma [8]. The cross reactivity or interference was secondarily investigated by determining the difference in ACTH recovery following room temperature storage during which degradation of the endogenous ACTH to fragments would occur. Recovery of [ACTH] was significantly higher for the CI assay than the IF assays on days 2-4, consistent with endogenous ACTH degradation products cross reacting with the CI assay or interfering with the IF assay. The true extent to which equine ACTH degrades under such conditions is unknown and it is not possible to determine which assay is more accurate for detection of intact ACTH.

The apparent difference between the cross reactivity/interference seen with samples from normal ponies and the lack of cross reactivity seen with the synthetic commercially available ACTH fragments could reflect altered immunoreactivity of the synthetic fragments compared with their endogenous counterparts. Such differences could result from sequence differences or post-translational modification such as glycosylation, acetylation, amidation and phosphorylation [14]. Partial sequence data for the equine POMC gene indicate some differences from human sequences, particularly for CLIP [15]. The use of recombinant peptides based on the equine POMC sequence

may have yielded different results. Alternatively, other POMC fragments, not analysed in the current study, could be responsible for the cross-reactivity/interference that is apparent *in vivo*. Further studies are warranted to explore these possible explanations.

Assuming cross-reactivity or interference occurred, the data in the current study indicate a seasonal effect on the concentration of the substance(s) responsible. Marked seasonal effects are described for the circulating concentrations of POMC derived peptides in the horse, in particular alpha-melanocyte-stimulating-hormone (α -MSH; POMC amino acids 1-13) [1,4,16]. The magnitude of autumnal increases in plasma α -MSH concentrations typically exceed those of ACTH and in one study, there was a more marked autumnal increase in ponies (11 fold increase) when compared with horses (2-fold increase) [17]. The seasonal effects on plasma concentrations of CLIP and other POMC derived peptides are not well reported in the horse, however an increase of similar magnitude to α -MSH would be expected for CLIP. Further, in PPID the concentrations of a range of POMC derived peptides may greatly exceed the increase in [ACTH] [18]. The equine pituitary secretome, and its seasonal variation in health and disease, is complex and assessment of plasma [ACTH] alone is likely to be a crude indicator of pituitary secretory activity. The current study suggests that unidentified changes in the pituitary secretome of clinically healthy ponies affects the performance of ACTH assays. It is unclear which assay has greatest diagnostic accuracy for PPID and further validation work of both assays in a population with a higher proportion of animals with clinical signs suggestive of PPID is required.

For clinicians that wish to use the IF assay, applying 24 pg/ml and 27 pg/ml as the upper limit of the reference range in the spring and autumn respectively to the ponies in this cohort produced good agreement with a published reference range for the CI analyser in the UK. Agreement for the ordinal classification of results as normal, equivocal or abnormal was weaker than for binary classification,

but still considered to be moderate. The improved recovery of ACTH when using the CI assay may also be relevant for clinicians if sample analysis is delayed. The high levels of ACTH recovery are consistent with other reports using the CI assay in the horse in which recovery is higher than would be expected in other species [2,19] such findings could be due to cross-reactivity of ACTH breakdown products.

A high proportion of ponies (64%) exceeded a published reference range (>47 pg/ml) in the autumn using the CI assay despite the relatively low prevalence of clinical signs, including an absence of laminitis, reported by owners or evident on veterinary examination. The majority (70%) of these ponies returned to within the seasonally adjusted reference range in the spring without receiving any treatment. It is unclear whether these findings indicate a very high burden of subclinical disease that is only revealed in the autumn, or an inappropriately low autumn diagnostic threshold. Early clinical signs of PPID may be subtle [13] and animals with early disease may have been overlooked. However many of the owners in the current study were very experienced and would be expected to be adept at distinguishing between normal and abnormal ponies. A very high diagnostic accuracy has been reported for diagnosis of PPID based on a higher basal [ACTH] threshold (>77 pg/ml) during the autumn [4], however autumn basal ACTH samples were only available from 4 cases that were considered PPID positive based on clinical signs. It is also suggested that ponies may have a greater seasonal increase in [ACTH] than horses [13] and a higher diagnostic threshold may therefore apply. Clinicians should interpret the results of basal ACTH testing with caution and with reference to clinical signs, larger equivocal diagnostic ranges may be required [6]. For horses that are diagnosed with PPID during the autumn, begin pergolide treatment and are then re-tested the following spring clinicians should be cautious before attributing a return of ACTH to reference range to pergolide treatment. Additional prospective clinical studies are warranted to determine the most appropriate diagnostic thresholds for ponies and their relationship with the presence of and development of clinical signs of PPID. It is currently unclear whether the IF or CI assay will have greater diagnostic

accuracy or whether specificity for ACTH is required for diagnosis of PPID. Cross reactivity to other POMC derived peptides produced by a dysfunctional *pars intermedia* could potentially result in improved diagnostic accuracy.

The current study had several limitations. Primarily, the true PPID status of the subjects was not determined. Insufficient numbers of ponies with definitive clinical signs were included in this largely healthy cohort and no histological testing or TRH stimulation tests were performed. The study therefore could not address whether either assay was superior to the other. Many clinical signs of PPID are non-specific and both veterinary and owner assessment of signs are highly subjective. The most biologically plausible explanation for the seasonally variable association between the assays was considered to be cross-reactivity/interference with unidentified POMC derived peptides in the autumn. A more rigorous analysis of this hypothesis would have required more sophisticated analysis of the autumnal pituitary secretome such as an HPLC-MS based approach and further work to address this question is warranted.

In conclusion, the CI and IF assays cannot be used interchangeably but good binary and moderate ordinal interpretation can be obtained using assay specific and season specific diagnostic thresholds.

The seasonal difference in the association between the assays suggests assay cross reactivity/interference to an altered autumnal pituitary secretome; however, the identity of the cross-reacting or interfering substance remains unknown and requires further investigation. Finally, a large proportion (70%) of ponies whose [ACTH] was above currently recommended reference ranges in the autumn had [ACTH] concentrations within the reference range the following spring without receiving any treatment for PPID.

Authors' declaration of interests

S. Shaw is employed by Tosoh Bioscience Ltd. E. Knowles is employed by CVS Group through which he provides services to Axiom Veterinary Laboratories. P. Harris is employed by WALTHAM/Mars Petcare.

Ethical animal research

The study was conducted under a Home Office licence and was approved by the Royal Veterinary College Ethics and Welfare Committee. Owners gave informed consent for their horses' inclusion in the study.

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Authorship

Study design was by E. Knowles, N. Menzies-Gow, J. Elliott, P. Harris and S. Shaw. Study execution was by E. Knowles and M. Moreton-Clack. Data analysis and interpretation was by E. Knowles, N. Menzies-Gow, P. Harris and J. Elliott. E. Knowles prepared the manuscript with contributions from N. Menzies-Gow, J. Elliott and P. Harris. All authors approved the final version of the manuscript.

Manufacturers' addresses

^aBecton Dickinson, Oxford, UK.

^bSiemens Healthcare, Sudbury, Suffolk, UK.

^cTosoh Bioscience, Redditch, Worcestershire, UK.

^dSigma Aldrich UK, Haverhill, Suffolk, UK.

^eMicrosoft, Redmond, Washington, USA.

^fIBM SPSS Statistics

Figure legends:

Fig 1: Box and whisker plots of ACTH concentrations measured with the CI or IF analysers in the Autumn or Spring. Whiskers indicate maximum and minimum values, box borders the first and third quartiles and the horizontal line within each box denotes the median value.

Fig 2: Scatter plots of [ACTH] measured using the CI and IF methods. Panel A shows all data. Panel B only includes data points with ACTH <100 pg/ml (using the CI assay) to indicate the different seasonal relationship within a similar range of values.

Fig 3: Apparent [ACTH] on addition of ACTH fragments.

Fig 4: Percentage recovery of ACTH following storage at room temperature. Significant differences between recovery with each assay ($p < 0.05$) are marked with asterisks.

Table 1: Commonly used chemiluminescent thresholds with corresponding values for the immunofluorescent and kappa statistics of agreement.

Season	Chemiluminescent threshold (pg/ml)	Immunofluorescent threshold (pg/ml)	Kappa (95% confidence intervals)
Non-autumn	>29	>24	0.74 (0.57-0.91)
	19-40 'grey zone'	18-24	0.52 (0.38-0.65)
Autumn	>47	>27	0.66 (0.51-0.80)
	>77	>33	0.69 (0.54-0.84)

Table 2: ACTH results using the CI assay with reference to commonly used diagnostic thresholds for the same ponies when tested in autumn 2015 and spring 2016.

Autumn 15	Spring 16
High (>47 pg/ml) N = 56	High (>29 pg/ml) N = 17
	Normal (\leq 29 pg/ml) N = 39
Normal (\leq 47 pg/ml) N = 32	High (>29 pg/ml) N = 3
	Normal (\leq 29 pg/ml) N = 29

References

1. McFarlane, D. (2011) Equine pituitary pars intermedia dysfunction. *Vet. Clin. North Am. Equine Pract.* **27**, 93-113.
2. Perkins, G.A., Lamb, S., Erb, H.N., Schanbacher, B., Nydam, D. V and Divers, T.J. (2002) Plasma adrenocorticotropin (ACTH) concentrations and clinical response in horses treated for equine Cushing's disease with cyproheptadine or pergolide. *Equine Vet. J.* **34**, 679-685.
3. Copas, V.E.N. and Durham, A.E. (2012) Circannual variation in plasma adrenocorticotropin hormone concentrations in the UK in normal horses and ponies, and those with pituitary pars intermedia dysfunction. *Equine Vet. J.* **44**, 440-443.
4. McGowan, T.W., Pinchbeck, G.P. and McGowan, C.M. (2012) Evaluation of basal plasma alpha melanocyte stimulating hormone and adrenocorticotrophic hormone concentrations for the diagnosis of pituitary pars intermedia dysfunction from a population of aged horses. *Equine Vet. J.* **45**, 66-73.
5. Rendle, D.I., Litchfield, E., Heller, J. and Hughes, K.J. (2013) Investigation of rhythms of secretion and repeatability of plasma adrenocorticotropin hormone concentrations in healthy horses and horses with pituitary pars intermedia dysfunction. *Equine Vet. J.* **46**, 113-117.
6. Rendle, D. (2017) Laboratory diagnosis of the endocrine causes of laminitis. *Livestock* **22**, 216-220.
7. Irvine, K.L., Burt, K., Hill, A.J., Shaw, S. and Pappasoulotis, K. (2016) Initial analytic quality assessment and method comparison of an immunoassay for adrenocorticotropin hormone measurement in equine samples. *Vet. Clin. Pathol.* **45**, 154-163.
8. Siemens Immulite ACTH package insert.
9. Giavarina, D. (2015) Understanding Bland Altman analysis. *Biochem. medica* **25**, 141-151.

- Accepted Article
10. Altman, D.G. and Bland, J.M. (1994) Statistics Notes: Diagnostic tests 3: receiver operating characteristic plots. *B. Med. J.* **309**, 188.
 11. Youden, W.J. (1950) Index for rating diagnostic tests. *Cancer* **3**, 32-35.
 12. Fleiss, J.L., Levin, B. and Myunghee, C.P. (2003) Measurement of Interrater Agreement. In: *Statistical Methods for Rates and Proportions*, Eds: J. Fleiss, B. Levin and C.P. Myunghee, John Wiley and Sons, Hoboken. pp 598-626.
 13. The Equine Endocrinology Group Recommendations for the Diagnosis and Treatment of Pituitary Pars Intermedia Dysfunction (PPID).
https://sites.tufts.edu/equineendogroup/files/2015/12/2015-10-16_EEG-2015-recommendations.pdf. Accessed June 1, 2016.
 14. Takahashi, A. and Mizusawa, K. (2013) Posttranslational Modifications of Proopiomelanocortin in Vertebrates and Their Biological Significance. *Front. Endocrinol. (Lausanne)* **4**, 143.
 15. Carmalt, J. (2017) Equine Pituitary Pars Intermedia Dysfunction (PPID): Pathophysiology and a surgical approach to treatment. p 81-113. PhD Thesis. University of Saskatchewan.
<https://ecommons.usask.ca/bitstream/handle/10388/7952/CARMALT-DISSERTATION-2017.pdf?sequence=1&isAllowed=y>. Accessed July 18th 2017.
 16. McFarlane, D., Paradis, M.R., Zimmel, D., Sykes, B., Brorsen, B.W., Sanchez, A. and Vainio, K. (2011) The Effect of Geographic Location, Breed and Pituitary Dysfunction on Seasonal Adrenocorticotropin and Alpha-Melanocyte-Stimulating Hormone Plasma Concentrations in Horses. *J. Vet. Intern. Med.* **25**, 872-881.
 17. McFarlane, D., Donaldson, M.T., McDonnell, S.M. and Cribb, A.E. (2004) Effects of season and sample handling on measurement of plasma alpha-melanocyte-stimulating hormone concentrations in horses and ponies. *Am. J. Vet. Res.* **65**, 1463-1468.

18. Wilson, M.G., Nicholson, W.E., Holscher, M.A., Sherrell, B.J., Mount, C.D. and Orth, D.N. (1982) Proopiomelanocortin peptides in normal pituitary, pituitary tumor, and plasma of normal and Cushing's horses. *Endocrinology* **110**, 941-954.
19. Rendle, D.I., Litchfield, E., Gough, S., Cowling, A. and Hughes, K.J. (2015) The effects of sample handling and N-phenylmaleimide on concentration of adrenocorticotrophic hormone in equine plasma. *Equine Vet. J.* **47**, 587-591.

Supplementary information items

Supplementary Item 1: Bland-Altman plot.

Supplementary Item 2: Apparent [ACTH] following addition of synthetic peptides to pooled plasma.

Table 1: Commonly used chemiluminescent thresholds with corresponding values for the immunofluorescent and kappa statistics of agreement.

Season	Chemiluminescent threshold (pg/ml)	Immunofluorescent threshold (pg/ml)	Kappa (95% confidence intervals)
Non-autumn	>29	>24	0.74 (0.57-0.91)
	19-40 'grey zone'	18-24	0.52 (0.38-0.65)
Autumn	>47	>27	0.66 (0.51-0.80)
	>77	>33	0.69 (0.54-0.84)

Table 2: ACTH results using the CI assay with reference to commonly used diagnostic thresholds for the same ponies when tested in autumn 2015 and spring 2016.

Autumn 15	Spring 16
High (>47pg/ml) N=56	High (>29pg/ml) N=17
	Normal (\leq 29pg/ml) N=39
Normal (\leq 47pg/ml) N=32	High (>29pg/ml) N=3
	Normal (\leq 29pg/ml) N=29





