Aus der Klinik für Pferde, Allgemeine Chirurgie und Radiologie
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Einfluss der Fütterung auf den ACTH Basalwert und den TRH Stimulationstest bei endokrinologisch gesunden und an Pituitary Pars Intermedia Dysfunction erkrankten Pferden

Inaugural-Dissertation
zur Erlangung des Grades einer
Doktorin der Veterinärmedizin
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Karolina Drożdżewska
Tierärztin aus Warschau (Polen)

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Tabellen und Abbildungen

Abkürzungsverzeichnis

α-MSH	α-Melanocorticotropin / α-Melanozyten-stimulierendes Hormon
Aug	August
ACTH	Adrenocorticotropin
AFP	Alpha-Fetoprotein
ALP	Alkalische Phosphatase
AMH	Anti-Müller Hormon
β-END	β-Endorphin
β-LPH	β-Lipotropin
bACTH	Konzentration des basalen Adrenocorticotropins
b/pACTH	Konzentration des basalen Adrenocorticotropins und Konzentration nach Stimulation mit Thyrotropin-Releasing-Hormon
BCS	Body Condition Score
CI	Konfidenzinterval
CLIP	Corticotropin-like intermediate Peptide
Dec	Dezember
EDTA	Ethylendiamintetraessigsäure
EMS	Equines Metabolisches Syndrom
EPO	Erythropoetin
Feb	February
GCT	Granulosazelltumor
Ig	Immunoglobulin
IMHA	Immunmediierte hämolytische Anämie
IMTP	Immunmediierte Thrombozytopenie
IQR	Interquartilabstand
Jan	January
Jun	Juni
NE	Norepinephrin
Nov	November
Oct	Oktober
pACTH	Konzentration des Adrenocorticotropins nach Stimulation mit Thyrotropin-Releasing-Hormon
POMC	Proopiomelanocortin
PPID	Pituitary Pars Intermedia Dysfunction
PTHrP	Parathormon-verwandtes Peptid
Sept	September

TK1	Thymidinkinase 1
TRH	Thyrotropin-Releasing-Hormon
TST	Thyrotropin-Releasing-Hormon Stimulationstest

1. Einleitung

Ein geriatrisches Pferd wird ein immer häufigerer Patient bei Tierärzten in Europa. Da die Prävalenz mehrerer Tumoren mit dem Alter steigt, stellen sie eine zunehmend wichtigere Differenzialdiagnose dar. Haut-Neoplasien, wie z. B. equines Sarkoid oder Melanom, sind gewöhnlich leicht zu erkennen. Im Gegensatz dazu sind innere Neoplasien eine diagnostische Herausforderung: die Symptome und klinisch-pathologischen Befunde sind oft unspezifisch und treten spät im Verlauf der Erkrankung auf, die Masse / Infiltration kann häufig nicht palpiert / dargestellt werden oder ist für eine Biopsie schwer zugänglich, was oft einen invasiven chirurgischen Eingriff zu weiterer Abklärung benötigt (z. B. Laparotomie oder Laparoskopie). Das Lymphom ist der häufigste bösartige, innere Tumor beim Pferd.

Tumormarker sind Hormone, Antikörper oder Enzyme, die durch manche neoplastischen Zellen produziert werden und zu paraneoplastischen Syndromen in Form von begleitenden klinischen Symptomen oder Auffälligkeiten in der Blutuntersuchung führen können. Die Messung der Konzentration oder Aktivität der Substanzen im Blut kann die Diagnose eines Tumors unterstützen, aber auch beim Monitoring und bei der Einschätzung des Therapieerfolges oder der Prognose behilflich sein. Leider ist die Produktion der Substanzen durch Tumorzellen inkonsequent, weshalb die Erkrankung nicht immer durch paraneoplastische Syndrome begleitet wird. Aus diesem Grund ist die Sensitivität der Tests oft unzufriedenstellend. Des Weiteren wird die Interpretation durch zahlreiche Einflussfaktoren, wie zum Beispiel Alter, begleitende Entzündung oder andere Erkrankungen erschwert. Außerdem ist die Anzahl bekannter und verfügbarer Tumormarker in der Pferdemedizin sehr begrenzt. Zu bekanntesten Beispielen gehören eine monoklonale Gammopathie (Überproduktion von Antikörpern) und die maligne Hyperkalzämie (Produktion von Parathormon-verwandtem Peptid) bei Lymphom oder Erythrozytose (Produktion von Erythropoetin) bei Hepatoblastom.

Die Pituitary Pars Intermedia Dysfunktion (PPID) ist neben dem Equinen Metabolischen Syndrom (EMS) die häufigste endokrinologische Erkrankung älterer Pferde, die durch ein Adenom oder Adenokarzinom der Pars Intermedia der Hypophyse hervorgerufen wird. Die neoplastisch veränderten melanotropen Zellen zeigen eine gesteigerte Hormonausschüttung – unter anderem Adrenocorticotropin (ACTH), das als Tumormarker zu PPID-Diagnostik eingesetzt wird. Bei der Interpretation müssen jedoch viele Einflussfaktoren, wie Jahreszeit, Stress, andere Erkrankungen, Alter, aber auch Farbe und Rasse des Pferdes berücksichtigt werden. Zudem ist die Sensitivität des basalen ACTH-Wertes (bACTH) niedrig und es kann zu falsch negativen Ergebnissen führen, besonders beim Frühstadium von PPID. Alternativ wird bei fraglichen Fällen ein Thyrotropin-Releasing-Hormon (TRH) Stimulationstest (TST)

empfohlen. Dabei wird die ACTH-Ausschüttung aus Melanotropen durch intravenöse Injektion eines TRH-Analogons induziert und zehn Minuten später gemessen (pACTH). Die Sensitivität des Tests ist höher als beim bACTH, aber es ist derzeit unklar, ob Body Condition Score (BCS), Rasse, Farbe und Alter des Patienten einen Einfluss auf pACTH haben.

Derzeit werden bACTH- sowie pACTH-Messung (b/pACTH) nur nach Heufütterung empfohlen, da vereinzelte Studien einen Einfluss von Kraftfutter und Fasten auf bACTH und/oder pACTH gezeigt haben. Es gibt jedoch eine Diskrepanz zwischen Publikationen und die anderen Autoren haben diesen Unterschied nicht entdeckt.

2. Literaturübersicht

2.1 Pituitary Pars Intermedia Dysfunktion

Die Pituitary Pars Intermedia Dysfunktion (PPID), früher auch als Equines Cushing Syndrom bezeichnet, ist eine der häufigsten endokrinen Erkrankungen älterer Pferde und Ponys (Mcgowan et al. 2013). Im Zuge der Erkrankung kommt es zu einer progressiven Degeneration der dopaminergen Neuronen im Hypothalamus, die eine inhibitorische Wirkung auf die Hypophyse haben. Der Dopamin-Mangel führt zu einer Hyperplasie bis hin zur Bildung von Adenomen oder Adenokarzinomen in der Pars intermedia der Hypophyse (Heinrichs et al. 1990; Boujon et al. 1993; Love 1993; Miller et al. 2008). Als Folge dessen kommt es zu vermehrter Ausschüttung von Peptiden aus den melanotropen Zellen der Pars intermedia. Hierbei handelt es sich um Proopiomelanocortin (POMC)-Spaltprodukte: Adrenocorticotropin (ACTH), α-Melanozyten-stimulierendes Hormon (α-MSH), Corticotropin-like intermediate Peptid (CLIP), β-Lipotropin (β-LPH) und β-Endorphin (β-END) (Orth und Nicholson 1982; Couetil et al. 1996; Castro und Morrison 1997). Im Gegensatz zu Menschen und Hunden und anders als vorher geglaubt wurde, führt ACTH aus der Pars intermedia nicht zu einer Aktivierung der Nebennierenrinde und eine Hyperkortisolämie ist beim Pferd mit PPID selten (Mcfarlane 2019). Nichtsdestotrotz ist das freie Cortisol im Blut höher als bei endokrinologisch gesunden Pferden (Hart et al. 2016).

Zu den typischen klinischen Symptomen einer fortgeschrittenen Erkrankung gehören Hypertrichose und Fellwechselstörung (Innera et al. 2013; Mcgowan et al. 2013; Menzies-Gow et al. 2023). Des Weiteren treten typischerweise Muskelatrophie, Fettumverteilung, abnormales Schwitzen, Lethargie und Leistungsschwäche auf (Aleman et al. 2006; Miller et al. 2008; Mcgowan et al. 2013; Banse et al. 2021; Kirkwood et al. 2022; Menzies-Gow et al. 2023). Zu häufigsten Komorbiditäten gehören Hufrehe, Immunschwäche (z. B. Wundheilungsstörung, rezidivierende Hornhautdefekte), Unfruchtbarkeit, Sehnen- / Bänderschwächer, Polyurie und Polydipsie (Karikoski et al. 2014; Hofberger et al. 2015; Karikoski et al. 2016; Tatum et al. 2020; Miller et al. 2021; Steel et al. 2022; Menzies-Gow et al. 2023). Die endokrinopatische Hufrehe wird durch eine Hyperinsulinämie verursacht (Asplin et al. 2007; De Laat et al. 2010). Um das Hufreherisiko besser einschätzen zu können, sollte bei jedem Pferd mit PPID eine Untersuchung auf Insulinresistenz durchgeführt werden (Hart et al. 2021). Hierbei können dynamische Tests, wie zum Beispiel der Oral Sugar Test mit PPID-Diagnostik verbunden werden (Hodge et al. 2019).

Zur Behandlung von PPID wird der Dopaminagonist Pergolid in der Dosierung 1 mg / Pferd oder 0,5 mg / Pony einmal täglich oral eingesetzt und die Dosierung sollte je nach klinischem Verlauf und Kontrollblutwerten nach Bedarf angepasst werden (Rendle et al. 2015b; Fortin et

al. 2020; Hart et al. 2021). PPID ist eine nicht heilbare Krankheit, aber mehrere Symptome können mit entsprechender Haltungsoptimierung und lebenslanger medikamentöser Therapie verbessert werden, weshalb eine frühzeitige Diagnosestellung von großer Bedeutung ist (Mcfarlane et al. 2017; Tatum et al. 2020; Miller et al. 2021; Steel et al. 2022).

2.1 Diagnostik

Die aktuelle Literatur beweist, dass zahlreiche früher empfohlene Tests, wie z. B. Kortisol-Bestimmung im Serum, Speichel oder Harn, nicht mehr zu PPID-Diagnosestellung geeignet sind (Van Der Kolk et al. 1994; Van Der Kolk et al. 2001; Hart et al. 2021). Hypertrichose und Fellwechselstörung sind stark verdächtig für PPID und können als pathognomische Symptome interpretiert werden. Nichtsdestotrotz treten sie erst spät im Verlauf der Erkrankung auf (Miller et al. 2008; Mcgowan et al. 2013). Eine histopathologische Untersuchung der Hypophyse gilt als Goldstandard, aber kann die Diagnose erst *post mortem* bestätigen (Miller et al. 2008). Auch die Bildgebung ist wegen der Notwendigkeit einer Vollnarkose und / oder Kontrastmittelapplikation nicht zur Routineuntersuchung geeignet (Pease et al. 2011).
Die Equine Endocrinology Group (Hart et al. 2021) empfiehlt heutzutage eine basale ACTH-Messung (bACTH) bei Pferden mit fortgeschrittenen klinischen Symptomen von PPID und den TRH-Stimulationstest bei fraglichen Fällen.

2.2.1 Basaler ACTH-Wert

Eine einmalige Messung von ACTH im Plasma zwischen Mitte November und Mitte Juli hat eine Sensitivität von 60% und eine Spezifizität von 87% (Menzies-Gow et al. 2023). Stress, andere Erkrankungen und akute Schmerzen verursachen eine Erhöhung des Wertes durch Stimulation der Hypothalamus-Hypophysen-Nebennieren-Achse (Stewart et al. 2019; Gehlen et al. 2020; Haffner et al. 2020). Da bei PPID-erkrankten im Gegensatz zu endokrinologisch gesunden Pferden keine tageszeitlichen Schwankungen des ACTH-Wertes vorliegen, kann die Messung zu jedem Tageszeitpunkt vorgenommen werden (Rendle et al. 2015a). Einen bedeutsamen Einfluss auf die ACTH-Konzentration hat jedoch die Jahreszeit, sowohl bei endokrinologisch gesunden als auch an PPID leidenden Pferden, mit Höchstwerten im Spätsommer und Herbst, weshalb man bei der Interpretation der Resultate jahreszeitabhängige Normwerte beachten sollte (Copas und Durham 2012; Durham et al. 2021; Hart et al. 2021; Durham et al. 2022). Zahlreiche Faktoren führen zu physiologischer Erhöhung des ACTH-Wertes zwischen Juli und November: Alter (Mcfarlane 2007; Durham und Shreeve 2017), primitive Rasse (Fredrick und Mcfarlane 2014; Durham und Shreeve 2017; Bamford et al. 2020; Potier und Durham 2020), weibliches Geschlecht (Durham und Shreeve 2017) und Schimmelfarbe (Durham und Shreeve 2017). Aus dem Grund ist die

Wahrscheinlichkeit, ein falsch positives Ergebnis im Spätsommer und Herbst zu bekommen erhöht (Mcfarlane 2019).
Des Weiteren spielt die Probebearbeitung eine entscheidende Rolle bei der ACTH-Bestimmung. Das Blut sollte in Ethylendiamintetraessigsäure (EDTA)-Blutröhrchen entnommen, durchgehend gekühlt, spätestens nach zwei Stunden zentrifugiert und innerhalb von zwei Tagen untersucht werden (Rendle et al. 2015c; Hart et al. 2021).
Die Equine Endocrinology Group (Hart et al. 2021) empfiehlt nur Heufütterung vor der basalen ACTH-Messung, weil Kraftfutter potenziell die Resultate erhöhen kann (Diez De Castro et al. 2014; Jacob et al. 2018).

2.2.2 TRH-Stimulationstest mit ACTH-Messung

Das Thyrotropin-Releasing-Hormon (TRH), auch Thyreoliberin genannt, ist ein Peptidhormon, welches physiologisch im Hypothalamus gebildet wird und sowohl an den thyreotropen und kortikotropen Zellen der Pars distalis als auch melanotropen Zellen der Pars intermedia der Hypophyse wirkt und eine Ausschüttung von POMC-Spaltprodukten verursacht (Mcfarlane et al. 2006; Beech et al. 2007; Beech et al. 2011b). Nach einer intravenösen TRH-Applikation steigt die Plasma ACTH-Konzentration bei allen Pferden an, der Anstieg fällt jedoch bei den PPID-Patienten signifikant höher aus (Mcfarlane et al. 2006; Beech et al. 2011a). Der TRH-Stimulationstest scheint zudem sensitiver als die Einzelmessung von ACTH zu sein und kann als ein geeigneter Test zur Identifizierung erkrankter Pferde im Frühstadium dienen (Beech et al. 2007; Funk et al. 2011).
Der TRH-Stimulationstest wird zwischen Juli und Dezember nicht empfohlen, weil es noch keine jahreszeitlich angepassten Referenzbereiche gibt, die Ergebnisse sind aber in dieser Zeit höher als im restlichen Jahr (Diez De Castro et al. 2014; Hart et al. 2021). Des Weiteren ist eine exakte Zeit der Blutentnahme nach Stimulation entscheidend und eine Verspätung von einer Minute kann bereits einen signifikanten Unterschied auf die Ergebnisse haben (Thane et al. 2022).
Restifo et al. (2016) haben festgestellt, dass es keine Unterschiede in der pACTH-Konzentration bei Pferden gibt, die gefüttert waren oder gehungert haben. Es wurden jedoch nur endokrinologisch gesunde Pferde untersucht. Außerdem bekam die gefütterte Gruppe nur Raufutter, weshalb ein Einfluss von Kraftfutter auf den TRH-Stimulationstest ungeklärt bleibt. Im Gegensatz dazu haben Diez De Castro et al. (2014) festgestellt, dass die Fütterung einen relevanten Einfluss auf das b/pACTH bei gesunden Pferden hat. Die Equine Endocrinology Group (Hart et al. 2021) empfiehlt daher derzeit, dass die Pferde für den TRH-Stimulationstest Heu ad libitum bekommen, jedoch kein Kraftfutter über zwölf Stunden.
Bis jetzt wurde der Einfluss der verschiedenen Futterbedingungen (gefastet / Heu ad libitum / Heu ad libitum mit Kraftfutter) auf die Ergebnisse des TRH-Stimulationstests zwischen

endokrinologisch gesunden und an PPID erkrankten Pferden nicht verglichen. Es ist derzeit auch weitgehend unklar, ob Faktoren, die bACTH-Konzentration beeinflussen bei der Interpretation des TRH-Stimulationstests ebenso berücksichtigt werden sollten. Da der TRH-Stimulationstest immer mehr an Bedeutung in der Früherkennung von PPID-Patienten gewinnt, ist es sinnvoll, möglichst viele Einflussfaktoren auf das Testergebnis zu identifizieren. Hauptziel der Studie war festzustellen, ob verschiedene Fütterungsbedingungen (Fasten / Heu ad libitum / Heu ad libitum mit Kraftfutter), aber auch eine graue Farbe, weibliches Geschlecht, Ponyrasse, Alter des Pferdes und Monat der Untersuchung einen Einfluss auf das b/pACTH haben. Hierbei sollten sowohl endokrinologisch gesunde als auch an PPID erkrankte Tiere untersucht werden. Daraus resultierend sollte die Aussagekraft und Vergleichbarkeit der Testergbenisse erhöht werden, um die Diagnostik von PPID-Patienten zu verbessern.

3. Publikation I – Markers for internal neoplasia in the horse

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REVIEW

WILEY

Markers for internal neoplasia in the horse

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Abstract

The diagnosis of internal neoplasia in horses is challenging. Increased production of hormones physiologic for adult animals (e.g., adrenocorticotropin, norepinephrine, and erythropoietin) or typical for the foetal phase (alpha-fetoprotein, anti-Müllerian hormone, and parathyroid-hormone-related protein) might aid in tumour diagnostics. Thymidine kinase-1 and alkaline phosphatase are examples of intracellular enzymes, whose activity in the blood may increase in some neoplasia cases. Furthermore, inappropriate production of abnormal monoclonal or autologous antibodies can accompany lymphoma and multiple myeloma. Many of those tumour markers lead to clinical or laboratory changes, called paraneoplastic syndromes, such as hypercalcaemia and erythrocytosis.

The interpretation of the results of the tumour marker measurements in horses is complicated due to many factors affecting the markers' concentration or activity (e.g., young age, pregnancy, and inflammation) and other diseases triggering the same changes. Moreover, the presence of paraneoplastic syndromes is inconsistent, which leads to low sensitivity of those substances as tumour markers.

In conclusion, screening for neoplasia in horses is not recommended. The measurement of tumour markers should be performed only in risk groups with suspicious clinical or laboratory findings, and the results should be interpreted with caution. It is advisable to add inflammatory markers to the tumour profile or repeat the measurements.

KEYWORDS
equine, horse, marker, neoplasia, tumour

1 | INTRODUCTION

The recognition of some integumentary neoplasia, such as melanoma, seems straightforward. Nevertheless, there is ongoing research on tests confirming the diagnosis in questionable cases or even predicting the development of some skin tumours, for example, a genetic test for a squamous cell carcinoma at the limbus in Haflingers (Lassaline et al., 2015) or a microRNA-based test for sarcoids (Cosandey et al., 2021). However, the detection of internal neoplasia is a lot more challenging. First, the clinical symptoms are usually unspecific (e.g., weight loss and fever), and there are only a few predilecting factors (e.g., metastatic melanomas in grey horses). Standard workup depends on the suspected localisation of the tumour and includes blood work, diagnostic imaging of the thorax/abdomen, rectal palpation and endoscopic examination. The use of advanced imaging modalities, such as computed tomography, has also been reported recently (Fouche et al., 2016). If possible, a biopsy or fine needle aspiration can be performed and subsequently examined histopathologically. Alternatively, a peritoneal or

pleural fluid can be examined cytologically, but both false-positive and -negative results are possible (Recknagel et al., 2012).

A routine blood examination is rarely specific but might indicate organ dysfunction caused by a tumour (e.g., liver failure due to hepatocellular carcinoma) or pathologic cell production (e.g., lymphocytosis due to lymphoma). Moreover, neoplastic cells can produce or induce the production of specific substances that cause secondary changes in blood work or clinical signs, which are called paraneoplastic syndromes. Measuring the level of those circulating substances can help to diagnose, determine the stage or prognosis, monitor treatment and control for the recurrence of neoplasia; thus, they are called tumour markers. Paraneoplastic syndromes reported in horses include pruritis and alopecia, hypertrophic osteopathy (Marie's disease), pemphigus, hypoglycaemia, hypercalcaemia, erythrocytosis, neuropathies, immune-mediated haemolytic anaemia (IMHA) or thrombocytopenia (IMTP), and monoclonal gammopathy (Axiak and Johnson, 2012; Möller, 2020). Only a few substances causing those changes are known, and even fewer can be measured in a horse. The aim of this review is to describe the circulating internal tumour markers available in equine medicine and the factors influencing their concentration or activity.

2 | HORMONES

2.1 | Adrenocorticotropin

Adrenocorticotropin (ACTH) is widely used as a marker of hormonally active adenoma or, less commonly, carcinoma of the pars intermedia of the pituitary gland in horses, which causes a clinical syndrome called pituitary pars intermedia dysfunction (PPID) (Equine Endocrinology Group, 2021; Heinrichs et al., 1990; Love, 1993). However, moderately higher levels of ACTH might also be caused by hyperplasia of the pars intermedia, and thus, this assay does not enable an exact diagnosis.

The pathognomonic clinical signs of PPID are hypertrichosis and a failure to shed the winter coat (McGowan et al., 2013b; Schott et al., 2017), but the risk of laminitis remains the most devastating complication of the disease (Karikoski et al., 2016). The lack of dopaminergic regulation from the hypothalamus results in the excessive production of proopiomelanocortin peptides, including ACTH, alpha-melanocyte-stimulating hormone, corticotropin-like intermediate lobe peptide and beta-endorphins by melanotropes. Measuring the alpha-melanocyte-stimulating hormone concentration was presumed to be more specific than ACTH for PPID diagnostic as the hormone is produced exclusively by melanotropes (ACTH can be produced by melanotropes and corticotropes), but it did not appear superior and is not commercially available (Beech et al., 2011b; McGowan et al., 2013a).

ACTH is released in the circadian rhythm in endocrinologically healthy horses, with the highest concentrations in the morning (Rendle et al., 2014). However, this fluctuation is absent in horses with PPID, and thus, the representative blood sample can be taken at any time of the day (Rendle et al., 2014). The ultradian rhythm is inconsistently detected, and a paired blood sample was not superior to the single timepoint ACTH measurement (Rendle et al., 2014). Nevertheless, the season strongly affects ACTH secretion, leading to the lowest concentrations in April (Durham, 2017) and the highest in late summer and autumn (Copas and Durham, 2012; Durham et al., 2021). The Equine Endocrinology Group (2021) recently recommended new reference values and cut-off values for four different times of the year (Table 1). The likelihood of detecting disease in autumn is increased (McGowan et al., 2013a); however, there are challenges to this assessment because physiologic variability is also magnified (McFarlane, 2019). Grey horses, mares and thrifty breeds, including ponies and donkeys, have a significantly higher increase in ACTH concentration in September (Bamford et al., 2020; Durham and Shreeve, 2017; Fredrick et al., 2014; Potier and Durham, 2020). Furthermore, the age-related rise in ACTH levels is highly pronounced in the autumn (Durham and Shreeve, 2017; McFarlane and Maxwell, 2017).

Although animals with severe pain have higher plasma ACTH concentrations (Stewart et al., 2019), they remain stable in patients with chronic, mild-to-moderate pain (Gehlen et al., 2020), which enables diagnostic in horses presented with diseases such as laminitis (Gehlen et al., 2020). However, testing in a visibly excited or stressed animal (e.g., after transport or during bad weather) is not recommended, as it causes a temporary release of ACTH into the circulation (Chapman et al., 2017; Haffner et al., 2020). Sedation with alfa2 agonists and butorphanol does not influence the concentration of basal ACTH within the first 10 min (Oberhaus et al., 2021). Moreover, horses receiving grain have higher basal ACTH levels; thus, patients are allowed to eat only hay before testing (Diez de Castro et al., 2014; Jacob et al., 2018).

The appropriate management of blood samples is crucial to obtain correct results. The ethylenediamine tetraacetic acid blood should be constantly cooled and centrifuged within 2 h (Stewart et al., 2020). The concentration of ACTH in the separated plasma remains stable over the course of 48 h (Stewart et al., 2020). Freezing of the sample should be avoided because the ACTH concentration already drops after one freeze–thaw cycle (Hu et al., 2020). Furthermore, the assay for measuring ACTH may lead to significant differences, and new reference values given by the Equine Endocrinology Group (2021) reflect lower results obtained by chemiluminescence commonly used nowadays (McGilvray et al., 2020).

To sum up, the ACTH concentration is not recommended as a screening test to detect neoplasia of the pituitary pars intermedia, and the results should always be interpreted together with clinical signs, age, breed, colour, sex, feeding and health status and excitement of the horse, as well as the season, sample management and assay (Equine Endocrinology Group, 2021) (Table 2). Based on the studies available, the measurement of a basal ACTH concentration has an average sensitivity of 84% and specificity of 97% (Beech et al., 2011a; Beech et al., 2007; van der Kolk et al., 1995). The thyrotropin-releasing hormone stimulation test with an ACTH measurement is more sensitive and enables the recognition of an early disease (Beech et al., 2011a; Beech et al., 2007; Beech et al., 2011b; Equine Endocrinology Group, 2021).

TABLE 1 Reported reference ranges and cut-off values for selected tumour markers (the values presented should only be used as a guideline and the laboratory's own reference ranges are recommended)

Marker	Reference range	Cut-off value	Literature
ACTH			
Dec–Jun		>40 pg/ml	Equine Endocrinology Group (2021)
July and Nov		>50 pg/ml	
Aug		>75 pg/ml	
Sep–Oct		>90 pg/ml	
10 min after TRH administration (Jan–Jun)		>200 pg/ml	
AFP			
	<10 ng/ml		Laboklin GmbH (2022)
AMH			
	<6.9 ng/ml		Renaudin et al. (2021)
		>10 ng/ml	Conley and Ball (2018)
Testosterone		>100 pg/ml	Conley and Ball (2018)
Inhibine		>0.8 ng/ml	Conley and Ball (2018)
EPO			
	8–25 mIU/ml		McFarlane et al. (1998)
	<9 mIU/ml		Jaussaud et al. (1994)
NE			
Calm	140–450 pg/ml		Yovich et al. (1984)
Excited	400–1200 pg/ml		
		>4× reference value	Fouche et al. (2016)
PTHrP			
	<1 pmol/L		Barton et al. (2004)
ALP			
	<450 IU/L		Laboklin GmbH (2022)
TK1			
	<2.2 U/L		Larsdotter et al. (2015)
	<1 pmol/min/ml		Wang et al. (2021)

2.2 | Alpha-fetoprotein

Alpha-fetoprotein (AFP) is a glycoprotein produced by the yolk sac and later by the liver of the mammalian fetus (Gitlin and Boesman, 1967). It is widely used to detect pregnancy abnormalities in human medicine (Bader et al., 2004). There is also extensive research on its use in equine obstetrics-the AFP level reaches a peak in the first trimester and decreases with the length of pregnancy (Vincze et al., 2015; Vincze et al., 2018). The AFP levels decline further postpartum over at least three weeks in healthy mares (Vincze et al., 2018) (Table 1) and over a minimum of one week in healthy foals (Prell, 2016).

The equine pregnancy abnormalities, including twinning, conception failure, placentitis and embryonic loss, cause increased AFP levels in the maternal serum (Canisso et al., 2015; Sorensen et al., 1990; Vincze et al., 2015; Vincze et al., 2018). Mizejewski (2015) discussed a possible role of human AFP as a positive inflammatory reactant in the prenatal phase or chronic viral hepatitis, and Prell (2016) reported significantly higher AFP levels in septic neonates in comparison to healthy ones.

Alongside the use of AFP in obstetrics, it is described as a liver tumour marker, as neoplastic cells originating from hepatocytes usually retain the ability to produce AFP (Tatarinov, 1964); thus, anti-AFP antibodies can be used in immunohistochemical staining (Wu et al., 1997) and therapy after conjugation with cytotoxic drugs (Hata et al., 1984; Kato et al., 1983).

Nevertheless, not all primary liver tumours produce AFP; they do not secrete it into the circulation or release it in a pulsatile manner (Beeler-Marfisi et al., 2010; Wu et al., 1997). Only 50 % of cases with hepatocellular carcinoma in human medicine have increased AFP levels in the blood (Bai et al., 2017; Song et al., 2016).

Primary liver tumours are extremely rare in equine medicine. Serum AFP was elevated in a case of hepatocellular carcinoma (Roby et al., 1990) and was within the reference range in a horse with hepatoblastoma (Beeler-Marfisi et al., 2010). Additionally, the authors

TABLE 2 Factors and diseases potentially affecting the activity or concentration of selected tumour markers

Marker	Tumour	Increase	Decrease/normal/absent
Hormones			
ACTH	Adenoma and adenocarcinoma of the pituitary pars intermedia	Hyperplasia of the pituitary pars intermedia, late summer and autumn, grey colour, ponies, age, stress, severe disease	Spring
AFP	Hepatoblastoma, hepatocellular carcinoma	Germ cell tumours, pregnancy, pregnancy abnormalities, foals, inflammation	Nonsecretory primary liver tumour
AMH	GCT	Intact males, other causes of ovary enlargement	Age >20 years (small number of antral follicles), early stage, nonsecretory tumour
EPO	EPO-producing tumours of liver and metastatic carcinomas	Excitement, chronic hypoxia (high altitudes, lung/cardiac diseases), kidney disease, doping	Polycythaemia vera
NE, normetanephrin	Pheochromocytoma	Stress, excitement	Nonfunctional pheochromocytoma
PTHrP	Lymphoma, ameloblastoma, carcinoma, multiple myeloma	Pregnancy, lactation, intact males, idiopathic systemic granulomatous disease (sarcoidosis)	
Enzymes			
ALP	Osteosarcoma, liver tumours	Foals and young horses, intestinal disorders, cholestasis	
TK1	Lymphoma, multiple myeloma	Inflammation, viral infections, kidney insufficiency	
Antibodies			
Autologous Ig against erythro-cytes/platelets	Lymphoma	Primary disease; secondary to bacterial or viral infection, drugs, toxins	
Monoclonal gammopathy	Multiple myeloma	Chronic inflammation on infection, chronic liver disease, lymphoma, other transient nonspecific antigenic stimulation	Nonsecretory multiple myeloma, early stage

detected high blood AFP levels in an Arabian filly with hepatoblastoma confirmed post-mortem. Unfortunately, little is known about the specificity of the marker, as it is not widely used in other hepatic diseases or inflammatory conditions. However, the authors' personal observation was that the AFP level was not increased in four horses with presumed pyrrolizidine alkaloid intoxication, including two end-stage liver failure cases.

The measurement of AFP is not recommended as a screening for liver tumours (Lennox et al., 2000), because many other factors, including individual variation, age, pregnancy, inflammation and other liver diseases, may change AFP levels in horses (Table 2). There are also reports of AFP-producing tumours of germ cells (mainly the yolk sac, ovaries and testis), the urinary tract, the stomach, the bile duct and the pancreas in human (El-Bahrawy, 2011).

Nevertheless, the AFP level can be a valuable adjacent diagnostic tool when suspecting a primary liver tumour in a young horse, as they were described only in the fetus (de Vries et al., 2013; Loynachan et al., 2007), neonates (Loynachan et al., 2007) and juvenile horses up to 4 years (Beeler-Marfisi et al., 2010; Gold et al., 2008; Lennox et al., 2000; Tirosh-Levy et al., 2019). The patients were usually diagnosed with liver dysfunction (Beeler-Marfisi et al., 2010; Gold et al., 2008; Tirosh-Levy et al., 2019) and were presented with ascites (de Vries et al., 2013; Loynachan et al., 2007) or pleural effusion (Axon et al., 2008), mucous congestion/erythrocytosis (Axon et al., 2008; Beeler-Marfisi et al., 2010; Gold et al., 2008; Lennox et al., 2000; Roby et al., 1990; Tirosh-Levy et al., 2019), persistent hypoglycaemia (Roby et al., 1990), pathologic fractures (Tirosh-Levy et al., 2019), haemolysis and icterus (Beeler-Marfisi et al., 2010).

2.3 | Anti-Müllerian hormone

Anti-Müllerian hormone (AMH) and inhibin are protein hormones produced by Sertoli cells in the male fetus and play a role in sex differentiation (Conley and Ball, 2018). Testes in adult stallions are still able to produce AMH; thus, its level can be used to differentiate between intact males, including cryptorchid animals, and geldings (Claes et al., 2013). In mares, AMH and inhibin are produced by granulosa cells surrounding antral follicles and are widely used as markers of granulosa cell tumour (GCT), the most common ovarian neoplasia in horses (Ball et al., 2008; McCue, 1998). An enlargement and multicystic appearance ('honeycomb') of the affected ovary are typically present in an ultrasound examination (Renaudin et al., 2021; Sherlock et al., 2016). Most animals are presented with abdominal pain (Renaudin et al., 2021; Sherlock et al., 2016), behavioural changes (stallion-like behaviour and aggression) and/or reproductive abnormalities (prolonged oestrus, nymphomania, and persistent anoestrus) resulting from disbalanced hormone production (Stabenfeldt et al., 1979). The most common of the latter are low serum progesterone concentrations (Ellenberger et al., 2007) and elevated levels of testosterone (40%–67% cases), inhibin (87%–90% cases) and AMH (Ball et al., 2013; Ball et al., 2008). The sensitivity of AMH depends on the cut-off value and lies between 98% and 100% (Ball et al., 2013; Murase et al., 2018). It is superior to inhibin (sensitivity of 80%), testosterone (sensitivity of 48%) or even their combination (sensitivity of 84%) (Ball et al., 2013; Murase et al., 2018). Based on the large study by Uliani et al. (2019), the new upper reference range for AMH was raised from 4.7 (Murase et al., 2018) to 6.9 ng/ml (Renaudin et al., 2021). The accuracy of AMH is >90 %, with a cut-off value of 10 ng/ml (Conley and Ball, 2018), owing to its sole production in the ovary, stability (long half-life time-36 h) (Conley and Ball, 2018) and unchanged concentration during the oestrus cycle or pregnancy (Conley and Ball, 2018; Uliani et al., 2019). However, the number of antral follicles and, thus, the age of the mare influence the serum AMH concentration, with variable values in young animals, high levels in mares aged 5–15 years and sinking concentrations after 20 years of age (Claes et al., 2015; Uliani et al., 2019). Additionally, increased AMH concentration was reported in other conditions causing transient enlargement of the ovary (e.g., haematoma) (Murase et al., 2018). Finally, false-negative results are possible in endocrinologically inactive GCT or the early stages of the disease, and it is recommended to repeat the measurement after 2–3 months if symptoms persist as the tumour is expected to grow (Conley and Ball, 2018). Alternatively, an additional measurement of inhibin and testosterone levels can be performed as a panel of hormones, which slightly increases the sensitivity (Conley and Ball, 2018). However, both inhibin and testosterone secretion increase during ovulation and pregnancy (Conley and Ball, 2018; Ginther et al., 2007); thus, further assessment of the progesterone and/or estrone sulphate level might be advantageous in their interpretation (Conley and Ball, 2018). Inhibin is a lot less variable than steroid hormones, and its accuracy is higher than 80% with a cut-off value of 0.8 ng/ml (Conley and Ball, 2018). Nevertheless, the specificity of the inhibin measurements was revealed to be low due to cross-reaction with its precursors (Robertson et al., 1996). The assay detecting isoform B of inhibin proved to be superior in GCT diagnostic (Conley et al., 2018; Ellenberger et al., 2007) and is widely available now. Testosterone is a steroid hormone secreted by granulosa-theca cells and by the adrenal cortex, which makes it the least accurate GCT marker, and only fairly high concentrations are likely to indicate GCT (Table 1) (Conley and Ball, 2018).

Complete excision of GCT can be confirmed endocrinologically because the hormone concentration drops to the reference range within a week (Conley and Ball, 2018).

2.4 | Erythropoietin

The renal cortex in adult horses and the liver in fetus are the primary sites of erythropoietin (EPO) production (Jaussaud et al., 1994). Hypoxia triggers EPO release, which stimulates the proliferation and differentiation of erythrocytes in the bone marrow and increases haemoglobin synthesis (Jaussaud et al., 1994). It might be misused as a doping agent as it improves the performance of athletes (Jaussaud et al., 1994).

Highly increased EPO levels lead to absolute erythrocytosis and congestion of the mucous membranes, which can be a paraneoplastic syndrome (Axiak and Johnson, 2012). A relative erythrocytosis due to excitement (spleen contraction), dehydration or measurement error should be initially ruled out in all cases (Berlin, 1975; Murray, 1966; Tirosh-Levy et al., 2019). The EPO levels were reported to be within normal limits or decreased in horses with primary erythrocytosis due to polycythemia vera, which can be confirmed by bone marrow aspiration (Beech et al., 1984; McFarlane et al., 1998; Munoz et al., 2009). Increased EPO concentrations (Table 1) might be physiologic (appropriate) or pathologic (inappropriate) (Axiak and Johnson, 2012; Berlin, 1975; McFarlane et al., 1998; Murray, 1966). Appropriate secondary erythrocytosis occurs due to EPO overproduction in patients with chronic tissue hypoxia (e.g., living at high altitudes, lung or cardiac diseases) (Tirosh-Levy et al., 2019). EPO can also be inappropriately produced in diseased kidneys (e.g., hydronephrosis or polycystic kidney disease) or by specific tumours (McFarlane et al., 1998). Hepatocellular carcinoma (Roby et al., 1990), hepatoblastoma (Gold et al., 2008) and metastatic carcinoma (Cook et al., 1995) all producing EPO were described in horses. However, there are two case reports of equine hepatoblastoma with presumed secondary erythrocytosis without increased EPO concentration (Lennox et al., 2000; Tirosh-Levy et al., 2019). The production of prostaglandin or another protein with EPO-like action instead of the hormone itself was discussed as a possible mechanism (Cook et al., 1995; John et al., 1997). The protective and anti-apoptotic roles of EPO on many cells, including neoplastic ones, were discovered recently and may explain the EPO production phenomenon in some tumours (Sytkowski, 2007).

The EPO seems a good marker, however, not a tumour-specific marker, which can be used in cases of erythrocytosis after the exclusion of other possible causes (doping, excitement, hypoxia, polycythaemia vera and kidney diseases). Paraneoplastic secondary erythrocytosis

seems more common than the primary one in horses (Beech et al., 1984; McFarlane et al., 1998).

2.5 | Norepinephrine and normetanephrine

Pheochromocytoma is a rare adrenal medullary tumour arising from chromaffin cells able to secrete catecholamines (Luethy et al., 2016). It is usually benign, unilateral and asymptomatic (Luethy et al., 2016; Yovich et al., 1984); however, when hormonally active, it can lead to an adrenergic crisis and severe colic-like signs, including tachycardia, tachypnoea, muscle tremor, sweating and anxiety (Luethy et al., 2016; Yovich et al., 1984). Hemoperitoneum after tumour rupture, myocardial lesions and multiple endocrine neoplasia has been described (Fouche et al., 2016; Luethy et al., 2016; Yovich et al., 1984). The most common clinicopathological findings are hyperglycaemia and hyperlactataemia (Fouche et al., 2016; Luethy et al., 2016; Yovich et al., 1984).

Yovich et al. (1984) described the measurement of blood norepinephrine as potentially diagnostic in horses, and the measurement of this hormone was the highest in the plasma and urine of a pony diagnosed with pheochromocytoma antemortem by Fouche et al. (2016). However, the measurement of adrenaline metabolites-normetanephrine and metanephrine-in plasma and urine is the test of choice in humans (Pappachan et al., 2014), and values higher than 4× the reference concentration are considered highly indicative of pheochromocytoma in people and dogs (Fouche et al., 2016; Salesov et al., 2015) (Table 1). Unfortunately, the reference range is not established in horses; thus, the results were compared to healthy controls, and only plasma normetanephrine and the urinary normetanephrine-to-creatinine ratio met these criteria in a clinical case (Fouche et al., 2016).

False-negative results are possible in nonfunctional pheochromocytomas and with inappropriate handling of the samples (blood needs to be taken in a chilled heparin tube, protected from light, stored at −80°C after centrifugation and separation of plasma; additional acidification of the urine is necessary) (Fouche et al., 2016; Salesov et al., 2015). More research study and clinical reports are necessary to obtain reference and cut-off values for pheochromocytoma diagnosis (Fouche et al., 2016).

2.6 | Parathyroid-hormone-related protein

Parathyroid-hormone-related protein (PTHrP) plays a central role in calcium homeostasis and skeletal formation in the fetus, and its concentration drops considerably in adults (Wysolmerski and Stewart, 1998). However, it is also necessary for transplacental calcium transport and mammary development; thus, its blood level is increased in pregnant and lactating mammals (Wysolmerski, 2012). Nevertheless, PTHrP levels are the highest in the milk due to its local production in the mammary gland, and it is considered an important factor in promoting calcium secretion in the milk and intestinal calcium absorption in the foal (Care et al., 1997).

Suva et al. (1987) discovered that circulating PTHrP and calcium concentrations increase in mammals with malignant neoplasia (pseudoparathyroidism/humoral hypercalcemia of malignancy), and it was described in horses with lymphoma (Mair et al., 1990; Marr et al., 1989), multiple myeloma (Barton et al., 2004), ameloblastoma (Rosol et al., 1994), squamous cell carcinoma (Karcher et al., 1990; Meuten et al., 1978), adrenocortical (Fix and Miller, 1987) and metastatic carcinoma (Cook et al., 1995). The PTHrP shares the amino-terminal region and acts through the same receptor as the prathyroid hormone, leading to excessive bone resorption, renal calcium reabsorption and phosphorus excretion (Suva et al., 1987). Neoplasia and humoral hypercalcemia of malignancy as a paraneoplastic syndrome should be considered in horses with hypercalcemia and hypophosphatemia after excluding renal disease (Toribio, 2011). Additional laboratory abnormalities associated with humoral hypercalcemia of malignancy include hypocalciuria, hyperphosphaturia, normal or decreased circulating PTH and increased PTHrP levels (Toribio, 2011). Reduced bone density and calcinosis (pathologic calcification of soft tissues) may occur over time (Toribio, 2011).

Human radioimmunoassay can be used to measure equine PTHrP in plasma or milk (Care et al., 1997; Ratcliffe et al., 1990), and the upper reference range is 1 pmol/L (Barton et al., 2004; Wysolmerski and Stewart, 1998) (Table 1). False-negative results are possible if measuring PTHrP in order to diagnose malignancy, as few other mechanisms were reported to result in hypercalcemia in those cases, for example, the production of interleukins, tumour necrosis factor or bone destruction due to tumour invasion (Barton et al., 2004; Van der Kolk, 2007). The specificity of the test is high because pathological concentrations are found almost only in horses with malignancies; however, high circulating PTHrP was also described in a pony with idiopathic systemic granulomatous disease (sarcoidosis) (Sellers et al., 2001).

3 | ENZYMES

3.1 | Alkaline phosphatase

Alkaline phosphatase (ALP) is produced by hepatocytes, biliary epithelial cells, the intestine and osteoblasts; thus, an increase in its activity in the blood can be indicative of cholestasis, intestinal pathology or bone remodelling (Hank et al., 1993). High values are physiologic in neonates due to increased bone isoenzyme activity, and they decrease significantly over the first 21 days of life to normalise at the age of 2–4 years (Hank et al., 1993). Furthermore, bone tumours, mainly osteosarcomas in horses (Bush et al., 2007), lead to bone destruction and can cause a pathologic increase in ALP activity and hypercalcemia. Van der Kolk (2007) highlighted the importance of the differentiation between hypercalcemia of malignancy and those tumours. The specificity of total ALP activity as a marker for osteosarcoma is poor, but the measurement of a bone-specific isoenzyme can increase it. Furthermore,

ALP can be used as a prognostic marker (Jenner, 2010), and its activity should normalise after surgical excision of the osteosarcoma.

3.2 | Thymidine kinase 1

Thymidine kinase 1 (TK1) is an intracellular enzyme that is activated in the G1 and S phases of the cell cycle when DNA doubles before mitosis or meiosis (Reichard and Estborn, 1951). There is a high production of this enzyme in fast-proliferating neoplastic cells, and increased serum activity is caused by the leakage of TK1 after their death (Hallek et al., 1992); thus, the TK1 activity in serum correlates with the proliferative activity of the tumour (Hallek et al., 1992). However, the enzyme is highly unspecific as it can be released from many cells, including hepatocytes, hematopoietic and various neoplastic cells (Moore et al., 2021; Tanaka et al., 1993).

The serum activity of TK1 is high in the human fetus and remains elevated in maternal blood up to the 39th week of gestation (Bieniarz et al., 1988). Increased TK1 activity is also described in patients with vitamin B12 deficiency and kidney failure due to the impaired excretion of the enzyme with urine (Hagberg et al., 1984). The effects of age, pregnancy, vitamin B12 deficiency and kidney failure have not been studied in horses so far.

Furthermore, a few viral infections in humans, mainly caused by the family *Herpesviridae* (e.g., herpes simplex virus, Epstein-Barr virus, varicella-zoster virus and cytomegalovirus) and hepatotropic viruses, are associated with the increased activity of serum TK1 (Gronowitz et al., 1984; Tanaka et al., 1993). There are no reports of serum TK1 activity in horses with equine herpesvirus infection or acute viral hepatitis. A link between equine herpesvirus-5 and lymphoma was described in a few case reports (Vander Werf and Davis, 2013; Vander Werf et al., 2014), and a potential cross-reactivity between cellular (equine TK1) and viral (herpes) thymidine kinase should be further examined.

Inflammatory diseases cause a temporary elevation in TK1 activity in humans and dogs, most probably due to the proliferation of the leucocytes and their lysis at the site of inflammation (Gronowitz et al., 1983; Nakamura et al., 1997). A modest increase in the serum activity of TK1 was detected in a few horses with various inflammatory conditions (Larsdotter et al., 2015; Moeller et al., 2020; Moore et al., 2021).

A few reports of utilising TK1 as a biomarker of hematopoietic tumours in horses (lymphoma and multiple myeloma) were quite promising (Larsdotter et al., 2015; Moeller et al., 2020; Wang et al., 2021), but other studies showed that its performance is below expectations because false-negative (Luethy et al., 2019; Moore et al., 2021) and false-positive results are reported (Moeller et al., 2020). Moore et al. (2021) described no significant difference between the TK1 activity in equids with inflammatory disorders, hematopoietic and other neoplasia. On the other hand, Wang et al. (2021) found that TK1 performed well in distinguishing between horses with suspected/confirmed lymphoma and tumour-free controls. Those discrepancies might be partially due to different assays used in both studies, with the method determining the TK1 activity using [^{3}H]-dThd as the substrate being more promising (Moore et al., 2021; Wang et al., 2021).

The reported sensitivity of TK1 as a tumour marker depends on the assay and corresponding cut-off value (Table 1) and lies between 71% and 74% (Larsdotter et al., 2015; Wang et al., 2021). There is a huge discrepancy between the specificity reported to be 14% in one study (Larsdotter et al., 2015) and 97% in another (Wang et al., 2021). Nevertheless, using TK1 as a single tumour marker is not recommended, but a serial measurement of the enzyme might be superior (Moeller et al., 2020; Nakamura et al., 1997). It is described as a reliable monitoring factor (i.e., to assess the efficacy of the therapy or predict relapse) in dogs and people (Gronowitz et al., 1983; Nakamura et al., 1997). Interpreting the course of TK1 activity rather than a single measurement might also allow the differentiation from inflammation or viral infection, as it should return to normal limits as soon as the inflammation/infection has ceased (Larsdotter et al., 2015) and it should remain elevated in real neoplasia cases. Furthermore, interpretation of the results together with clinical findings and inflammatory markers (e.g., leucocyte count and serum amyloid A) might be useful (Larsdotter et al., 2015; Nakamura et al., 1997); however, an unspecific inflammatory reaction usually accompanies neoplasia (Winter et al., 2014).

4 | ANTIBODIES/IMMUNOGLOBULINS

4.1 | Autologous antibodies against erythrocytes and/or platelets

Both IMHA and IMTP were reported as a paraneoplastic syndrome in equine lymphoma cases (McGovern et al., 2011; Reef et al., 1984), and neoplasia is more common in horses with IMHA/IMTP than in healthy controls (Easton-Jones et al., 2021). However, none of the 15 lymphoma cases described in the retrospective study by Luethy et al. (2019) had IMHA or IMTP.

Autologous antibodies can be detected by flow cytometry (Davis et al., 2002). Furthermore, immunoglobulins (Ig) against erythrocytes can be detected in the serum by an indirect Coombs test or on the erythrocytes' surface by an direct Coombs test (Easton-Jones et al., 2021). The results should be interpreted with caution because pathologic production of autologous Ig can be a primary or secondary condition triggered not only by neoplasia but also by drugs, toxins, bacterial (e.g., *Streptococcus* spp., *Clostridium* spp., and *Leptospira* spp.) or viral (equine infectious anaemia) infections (Easton-Jones et al., 2021).

4.2 | Monoclonal gammopathy

Multiple myeloma is a multifocal or diffuse malignant proliferation of plasma cells within the bone marrow or in extraosseous sites (e.g., lymph nodes, kidney, spleen) (Barton et al., 2004; Eberhardt et al., 2018; Edwards et al., 1993). A clone of Ig or its fragments (usually a light chain) is called a paraprotein or M-component. They are produced in

excess by neoplastic cells, causing hyperproteinaemia and a sharp peak in serum and/or urine electrophoresis (Eberhardt et al., 2018; Edwards et al., 1993). Monoclonal paraproteins can be detected in the alpha2, beta or gamma region, and they may belong to class IgG or IgA (Barton et al., 2004; Eberhardt et al., 2018; Edwards et al., 1993; Kent and Roberts, 1990).

The most consistent laboratory findings include hyperproteinaemia due to hyperglobulinaemia, hypoalbuminemia, anaemia and proteinuria (Eberhardt et al., 2018; Edwards et al., 1993); thus, multiple myeloma should be suspected in horses with those changes. If normal, serum electrophoresis should be repeated in suspicious cases as typical results might not be detected in the early course of the disease (Barton et al., 2004). Nevertheless, even serial tests might lead to false-negative results in nonsecretory tumours (Edwards et al., 1993). Furthermore, chronic inflammation or infection, chronic liver disorders, other neoplasia and other antigenic stimulation lead to polyclonal gammopathy, which may complicate the diagnosis of a monoclonal increase of Ig (Barton et al., 2004; Eberhardt et al., 2018). Monoclonal gammopathies were also described in horses with lymphoma (Traub-Dargatz et al., 1985) or with transient, benign disorders, which further highlighted the importance of repeated measurements (Kent and Roberts, 1990).

Radial immunodiffusion is another method allowing the classification of Ig and supporting the diagnosis of multiple myeloma in questionable cases (Barton et al., 2004). Bence-Jones proteinuria can be present because overproduced light chains of Ig can be deposited in organs as AL-amyloid (Kim et al., 2005) or secreted in the urine as Bence-Jones bodies (Edwards et al., 1993). Eberhardt et al. (2018) recommended the human guidelines for the diagnostic of multiple myeloma in horses, which include detection of > 10% of plasma cells in a bone marrow biopsy or an extramedullary plasmacytoma and more than one of the following abnormalities: hypercalcaemia, kidney insufficiency, anaemia or bone lesions (Rajkumar et al., 2014).

5 | SUMMARY

There are three main groups of internal tumour markers in equine medicine: hormones, enzymes (TK1, ALP) and antibodies (monoclonal gammopathy, autologous Ig against erythrocytes or thrombocytes). Unfortunately, none of the substances described is an ideal biomarker, and general screening for neoplasia in horses is not recommended. The results should be interpreted together with history, signalment, suspicious clinical signs or laboratory findings (i.e., paraneoplastic syndromes) and evaluated for consistency. Many factors increase the concentration/activity of the tumour markers reported (Table 2), and the most common are young age, pregnancy, lactation, inflammation, infection or severe disease. Interpretation of the results simultaneously with inflammatory markers can help to avoid false-positive results, and tumour profiles usually contain them. Although inflammation often accompanies neoplasia, the values should normalise when the inflammation ceases, therefore serial measurements are recommended. Furthermore, tumours usually progress, and thus, the results of repeated tests remain elevated or increase.

More research needs to be undertaken to clarify the sensitivity and specificity of tumour markers in equine medicine.

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ORIGINAL ARTICLE

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Influence of feeding and other factors on adrenocorticotropin concentration and thyrotropin-releasing hormone stimulation test in horses and ponies

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Abstract

Background: The basal (bACTH) and post-thyrotropin-releasing hormone stimulation concentration of adrenocorticotropin (pACTH) are recommended for diagnosis of pituitary pars intermedia dysfunction (PPID). Many factors influence bACTH (e.g., disease, age, month) and some affect the results only in autumn (e.g., breed, colour, sex). There are discrepancies about the impact of feeding on b/pACTH.

Objectives: To determine whether feeding, month, age, breed, colour, sex and body condition score affect b/pACTH.

Study design: Prospective crossover.

Methods: Sixty-one animals were divided into groups: healthy, PPID, treated-PPID. The b/pACTH was measured three times (1 mg protirelin; blood collection after 10 min; mid-November to mid-July) after different feedings: fasting, hay, hay + grain. Friedman's test was applied to evaluate the influence of feeding on b/pACTH and linear mixed model to evaluate impact of further factors.

Results: The b/pACTH was not significantly affected by feeding ($p = 0.7/0.5$). The bACTH was lowest in healthy (29.3 pg/mL, CI 9–49.5 pg/mL) and highest in PPID-group (58.9 pg/mL, CI 39.7–78.1 pg/mL). The pACTH was significantly lower in healthy (396.7 pg/mL, CI 283.2–510.1 pg/mL) compared to PPID (588.4 pg/mL, CI 480.7–696.2 pg/mL) and treated-PPID group (683.1 pg/mL, CI 585.9–780.4 pg/mL), highest in July (881.2 pg/mL, CI 626.3–1136.3 pg/mL) and higher in grey (723.5 pg/mL, CI 577.5–869.4 pg/mL) than other colours (338.7 pg/mL, CI 324.8–452.5 pg/mL). The size of effect for those variables was >0.5.

Main limitations: Small number of animals, subsequent bACTH measurements were significantly lower in each horse.

Conclusions: There was no evidence that feeding influences the b/pACTH. There was evidence that pergolide affects the bACTH but it had little effect on pACTH. Further investigation of the impact of month and coat colour on b/pACTH is warranted to better interpret the results.

KEYWORDS
ACTH, adrenocorticotropin, endocrine, horse, PPID, TRH

1 | INTRODUCTION

Generalised hypertrichosis is considered a pathognomonic clinical sign in horses with pituitary pars intermedia dysfunction (PPID) and treatment without further testing is considered appropriate.[1,2] Unfortunately, changes in haircoat are usually manifested in the late stage of the disease and give no measure of the severity.[3] The measurement of the basal adrenocorticotropin concentration (bACTH) and thyrotropin-releasing hormone stimulation test (TST) with ACTH measurement (post-stimulation ACTH concentration: pACTH) are the most accurate diagnostic methods for PPID currently described.[4] The TST shows better than bACTH accuracy reaching 97% and is preferred for early case detection.[1,5–8] TST is recommended after feeding hay only[1] as grain was described to increase[9] and fasting to decrease the b/pACTH concentration.[10,11] Feeding was also described to increase bACTH secretion and consequently the cortisol concentration in other mammals.[12] However, studies are inconsistent and other authors showed no differences in TST results between fasted and fed horses.[11]

There are generally many factors known to affect the bACTH, but only a few have been described for the pACTH. The bACTH is influenced by the time of the year,[13,14] thus, there are season-specific reference ranges facilitating the interpretation.[1] The TST is not recommended in the late summer and autumn as the normal values have not yet been reliably studied.[1,14] Ageing, severe disease, acute pain and stress also increase the bACTH.[15–18] The significant effect of breed, colour and sex on the bACTH was also shown, with ponies, grey horses and mares having a higher bACTH in the autumn.[17,19] Studies investigating the influence of the body condition score (BCS) on the bACTH are lacking, however, it warrants further investigation as obesity and appetite correlates with dopaminergic tone in humans.[20] To the best of our knowledge, the effects of the above-mentioned factors on the pACTH have not been studied.

The aim of this study was to determine whether feeding 12 h prior to testing affects the bACTH and pACTH in endocrinologically healthy horses and ponies and those with PPID between mid-November to mid-July. Additionally, the influence of the month of testing, pony breed, grey colour, female sex, BCS and age were studied. We hypothesised that (1) feeding and age will affect the bACTH and pACTH, but (2) the month of testing, pony breed, grey colour, female sex and BCS will not affect the results of either test in the non-autumn months.

2 | MATERIALS AND METHODS

2.1 | Animals

The animals were enrolled in the study by their owners voluntarily, upon signing the consent form for the animal use in the research project. Clinically healthy horses and ponies of all breeds, older than 16 years, were included in the study. The breed, sex, age, colour, clinical signs and treatment were documented. Ponies were defined as animals with a certain pedigree (e.g., Shetland pony, Welsh A, German riding pony) or ponies of mixed breed measuring less than 148 cm at the withers (according to the International Federation of Equestrians) and weighing less than 250 kg. The weight was obtained by scale or was estimated using a measuring band (The Laminitis Clinic Weightape, EquiLife). The BCS (1–9) was assessed according to Henneke et al.[21]

Animals were divided into three groups based on clinical signs, treatment with pergolide and the bACTH (seasonally adjusted reference values from the Equine Endocrinology Group (2019)[22] were applied): (0) healthy, (1) PPID or (2) PPID treated with pergolide. There were horses and ponies in the healthy group without clinical signs typical of PPID (hypertrichosis and laminitis as exclusion criteria) and normal or questionable results of the bACTH (<50 pg/mL). Animals in the PPID group showed hypertrichosis and/or delayed shedding as an owner complaint or during the examination, and at least one more clinical sign described in PPID cases (e.g., polyuria/polydipsia, muscle loss, increased sweating, fat deposits, lethargy, susceptibility to infections).[2,23–25] The bACTH was increased or questionable (>30 pg/mL). Equids in the treated-group were diagnosed with PPID based on the bACTH or pACTH in the past and received pergolide mesylate (Prascend, Boehringer Ingelheim or Pergoquin, WDT) orally once daily for at least 4 weeks. The dosage was individually chosen for each case.

2.2 | TRH stimulation test

A short clinical examination (heart and respiratory rate, temperature) was performed before all tests. Each animal received 1 mg of protirelin (TRH Ferring 0.2 mg/mL, Ferring GmbH) intravenously and was observed for 30 min afterwards. All side effects were documented. The blood was collected in tubes with ethylene diamine tetraacetic acid just before and exactly 10 min after the injection. The samples

were kept in a cooling box with ice packs (not refrigerated) and centrifugated within 2 h. The ACTH was measured in cooled plasma within 24 h by an external laboratory (Laboklin Laboratory for Clinical Diagnostics GmbH &Co KG) with Immulite 2000™ (Siemens Healthcare Diagnostics; solid-phase, two-site sequential chemiluminescent immunometric assay with analytic sensitivity: 5 pg/mL, intraassay coefficient of variation: 2.97, interassay coefficient of variation: 8.92%).[26] All tests were performed between mid-November and mid-July[22] in the morning hours (5–12 AM). The results were interpreted according to the recommendation of the Equine Endocrinology Group from 2019.[22]

2.3 | Study design

The study was based at an equine clinic in Germany (latitude of 52.520008°N). It had a prospective, crossover design and each animal underwent three TSTs after different overnight feeding protocols: (A) hay ad libitum, (B) hay ad libitum + grain (oat, 2% of the bodyweight, 2 h prior to testing), or (C) fasting (muzzle or box with shavings). The order was stratified randomised to obtain equal number of feeding protocol sequences in each group (blind selection of an envelope with one of the six possible feeding orders for each horse; the envelopes were reused every six horses). At least 2 weeks of washout period was provided between the tests. Animals were allowed to stay at home and perform at the usual level.

Sample size calculation was done for bACTH. The hypothesis tested was that the feeding (independent variable; fasting, hay, hay + grain) affects the bACTH (dependent factor). The examination of 20 healthy, 20 with PPID affected and 20 treated-PPID animals allows to detect the differences of 2× standard deviations with a certainty of 95% and the power of 95%.

2.4 | Data analysis

The statistical analysis was performed separately for the bACTH and pACTH using IBM® SPSS Statistics Version 29 (SPSS Inc.). The analysis of variance (related-samples Friedman's two-way analysis of variance by ranks) was applied to examine the influence of the feeding on the tests in each group. In the linear mixed models, horse, interaction horse*group and horse*feeding were included as random effects, while group, feeding, test order, month of testing, BCS, breed (pony effect), sex (mare effect), colour (grey effect), age and the interaction group*feeding were fixed effects. The b/pACTH were dependent variables. Bonferoni test was used as a post hoc analysis. The level of significance was set at $p < 0.05$. Normality and homoscedasticity of residuals were checked by visual inspection. Since the interactions of horse*group and horse*feeding were not significant, they were removed leaving horse as random effect in the final model. The effect size of variable was calculated, where appropriate.[27] The assay upper limit of detection of ACTH in the external laboratory selected was 1250 pg/mL, thus, the higher results were changed to 1251 pg/mL to facilitate the statistical analysis.

3 | RESULTS

Sixty-two examinations were performed between January 2020 and February 2022. One horse from the healthy group underwent only two out of three TSTs and was lost for the third test due to COVID-19 restrictions, therefore, it was excluded from the study. As a result, 61 examinations consisting of three TSTs each, were included (Table 1). There were 53 animals, as 8 horses were enrolled in the study twice—they started in healthy group and were included in the treated-group after the treatment with pergolide. The duration of treatment with pergolide in the treated-PPID group was recorded for 18/20 animals (two were treated for longer than a year and the owners did not provide a beginning date) and the average was 233 days (range 29–1177 days). The mean dosage of pergolide was 0.7 mg (range 0.25–2 mg). There were 33 horses and 20 ponies, 22 mares and 31 geldings, 6 grey horses and 47 of different colours. The average BCS and the average age were similar between groups (Table 1).

The TST was performed successfully in all horses. However, in two uncooperative animals there was a delay of up to 1 min in the blood collection after TST. Three animals had one pACTH result outside the detectable limit and one horse had pACTH >1250 pg/mL after all feedings, so there were six measurements above the detectable limit. All bACTH were <1250 pg/mL.

The side effects observed included sneezing (27.9%), lip smacking (27.3%), itching (rubbing the nose or rolling; 21.3%), passing faeces (18%, including 9.8% single episodes of loose faeces), flatulence (16.4%), pawing (15.3%), urination (12.0%), yawning (11.5%), coughing (10.9%), shaking out (8.7%), flehmen response (7.7%) and trembling (2.7%). Most of the observations were noticed and disappeared within 10 min. One horse showed mild colic signs 7 h after the test, which resolved after an injection of metamizole (15 mg/kg intravenously; Novaminsulfon, 500 mg/mL, bela-pharm GmBH & Co. KG).

3.1 | bACTH

The data sets were not normally distributed, thus, the data are presented as median + interquartile range (IQR). There were no differences in the bACTH after different feeding protocols in all groups applying the related-samples Friedman's two-way analysis of variance by ranks ($p = 0.7$; fasting: 24.5 pg/mL, IQR 19.0–33.6 pg/mL; hay: 24.0 pg/mL, IQR 17.8–32.5 pg/mL; hay + grain: 24.2 pg/mL, IQR 17.8–32.7 pg/mL) (Figure 1).

The residuals were normally distributed in the linear mixed model, thus, the results are presented as mean ACTH + 95% confidence interval (CI). The bACTH was significantly affected by the group ($p = 0.02$) and order of the tests ($p = 0.03$). The feeding protocol ($p = 0.4$), month of testing ($p = 0.5$), BCS ($p = 0.3$), pony breed

TABLE 1 Descriptive statistic including the number, age, BCS, sex, breed and colour of the animals included in each group in the study (healthy, PPID, treated PPID).

Group		0 (healthy)	1 (PPID)	2 (treated-PPID)
Number		20	21	20
Age in years	Mean ± SD	22.0 ± 4.5	24.2 ± 4.2	24.9 ± 5.3
	Range	16–31	17–33	16–35
BCS	Median	6	5	6
	Range	4–8	4–8	3–8
Sex	Mares	8	11	9
	Geldings	12	10	11
	Stallions	0	0	0
Breed	Ponies	10	3	7
	Horses	10	18	13
Colour	Grey	0	4	4
	Other	20	17	16

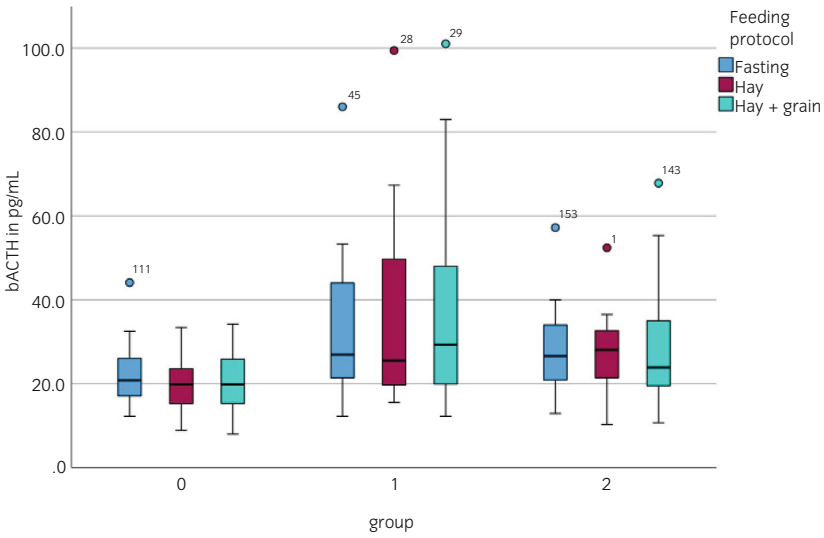

FIGURE 1 Cluster box plot of the bACTH by group (0—healthy, 1—PPID, 2—treated-PPID) and feeding protocol (fasting, hay, hay + grain).

($p = 0.8$), female sex ($p = 0.3$), grey colour ($p = 0.3$), age ($p = 0.3$) and interaction group*feeding protocol ($p = 0.3$) were not significant in the presented model.

Horses in the PPID-group (58.9 pg/mL, CI 39.7–78.1 pg/mL) had significantly higher bACTH ($p = 0.03$, Cohen's $d = 0.7$) than healthy animals (29.3 pg/mL, CI 9–49.5 pg/mL). The mean difference (mdf) was 29.7 pg/mL (1.9–57.4 pg/mL). The bACTH was not significantly ($p = 0.5$, Cohen's $d = 0.5$) higher in PPID-group than in the PPID-treated group (32.9 pg/mL, 15.6–50.1 pg/mL).

The first bACTH measurement was significantly higher than the last one in each animal ($p = 0.03$, Cohen's $d = 0.3$). The mean difference was 15.0 pg/mL (CI 1.3–28.7 pg/mL). The mean bACTH was 47.6 pg/mL (32.1–63.0 pg/mL), 40.8 pg/mL (25.4–56.4 pg/mL), 32.6 pg/mL (CI 17.2–48.0 pg/mL) in the first, second and third test respectively.

The mean bACTH was highest in July (47.3 pg/mL, CI −2.9 to 97.6 pg/mL) and lowest in May (31.6 pg/mL, CI 8.4–54.7 pg/mL). Mares (45.2 pg/mL, CI 29.7–60.7 pg/mL) had not significantly higher bACTH ($p = 0.3$, Cohen's $d = 0.4$) than geldings (35.5 pg/mL, CI 17.3–53.6 pg/mL) and grey horses (47.1 pg/mL, CI 21.2–72.9 pg/mL) higher bACTH ($p = 0.3$, Cohen's $d = 0.4$) than other coat colours (33.6 pg/mL, CI 22.1–45.2 pg/mL).

3.2 | pACTH

The data sets were not normally distributed, thus, the data are presented as median + IQR. There were no significant differences in the pACTH between feeding protocols in all groups applying the related-samples Friedman's two-way analysis of variance by ranks ($p = 0.5$; fasting: 240.0 pg/mL, IQR 94–625.5 pg/mL; hay: 233.0 pg/mL, IQR 80.5–563.0 pg/mL; hay + grain: 273.0 pg/mL, IQR 94.6–571.5 pg/mL) (Figure 2).

The residuals were normally distributed in the linear mixed model, thus, the results are presented as mean pACTH + CI. There was a significant influence of the group ($p < 0.001$), month of testing ($p < 0.001$), colour ($p < 0.001$) and age ($p = 0.002$). The pACTH was

not significantly impacted by feeding ($p = 0.7$), BCS ($p = 0.9$), pony breed ($p = 0.6$), female sex ($p = 0.1$) and interaction group*feeding ($p = 0.9$) in the presented model. The pACTH was significantly lower ($p = 0.01$, Cohen's $d = 1.2$) in the healthy group (396.7 pg/mL, CI 283.2–510.1 pg/mL) compared with PPID (588.4 pg/mL, CI 480.7–696.2 pg/mL) and significantly lower ($p < 0.001$, Cohen's $d = 1.7$) compared with treated-PPID (683.1 pg/mL, CI 585.9–780.4 pg/mL). The mean pACTH was 286.5 pg/mL (CI 137.6–435.3 pg/mL) higher in PPID-treated group than in healthy animals. The pACTH was not significantly higher in treated-PPID group compared with untreated ($p = 0.4$, Cohen's $d = 0.4$; mdf 94.7 pg/mL, CI −52.0 to 241.5 pg/mL).

The pACTH was the highest in June (626.0 pg/mL, CI 486.3–765.6 pg/mL), July (881.2 pg/mL, CI 626.3–1136.3 pg/mL) and November (682.7 pg/mL, CI 539.0–826.5.9 pg/mL) and the lowest in April (408.2 pg/mL, CI 297.0–519.5 pg/mL) (Figure 3). The pACTH was significantly higher in November compared with January ($p = 0.01$, Cohen's $d = 1$; mdf 227.1 pg/mL, CI 25.4–429.0 pg/mL), in July compared with April ($p = 0.02$, Cohen's $d = 3.3$; mdf 473.1 pg/mL, CI 38.5–907.7 pg/mL), in November compared with April ($p = 0.03$, Cohen's $d = 1.4$; mdf 274.5 pg/mL, CI 14.1–534.9 pg/mL) and in November compared with December ($p = 0.01$, Cohen's $d = 1.2$; mdf 231.7, CI 23.7–439.6 pg/mL).

Furthermore, the pACTH was significantly higher ($p < 0.001$, Cohen's $d = 1.9$) in grey horses (723.5 pg/mL, CI 577.5–869.4 pg/mL) compared with other colours (338.7 pg/mL, CI 324.8–452.5 pg/mL). The mean difference was 334.8 (CI 174.6–495.0 pg/mL).

The first pACTH measurement (593.5 pg/mL, CI 508.7–678.3 pg/mL) was not significantly higher ($p = 0.1$, Cohen's $d = 0.2$) than the second (540.9 pg/mL, CI 455.9–625.9 pg/mL) and higher ($p = 0.1$, Cohen's $d = 0.2$) than the third one (533.8 pg/mL, CI 449.2–618.4 pg/mL) in each horse.

Ageing influenced the results significantly, however, the mean decrease per year was trivial and not biologically relevant. However not statistically significant ($p = 0.1$, Cohen's $d = 0.4$), mares had higher pACTH than geldings (mares: 597.2 pg/mL, CI

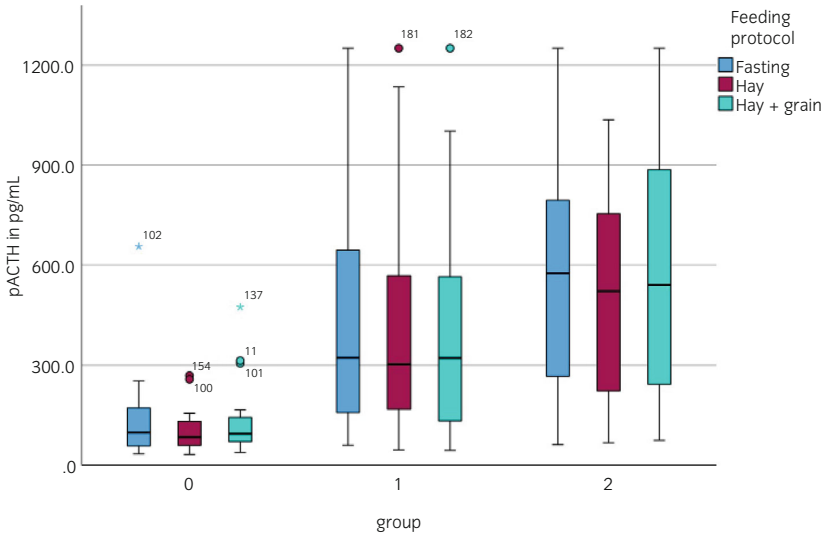

FIGURE 2 Cluster box plot of the pACTH by group (0—healthy, 1—PPID, 2—treated-PPID) and feeding protocol (fasting, hay, hay + grain).

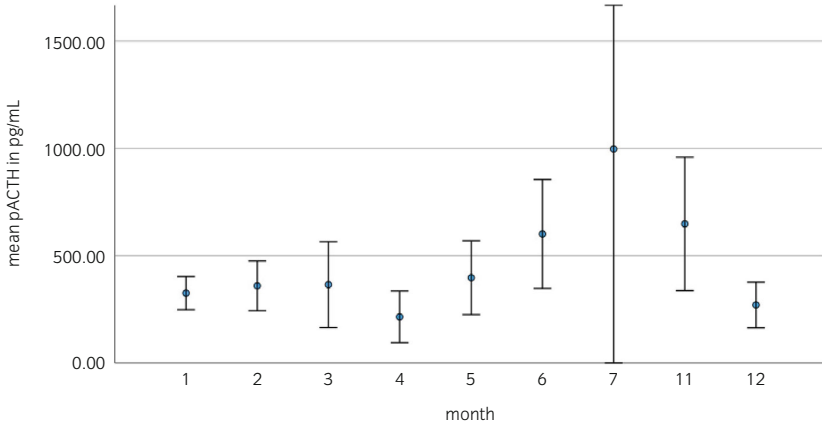

FIGURE 3 Chart with mean pACTH (pg/mL) with error bars presenting 95% confidence interval by month. The study was not conducted between August and October.

510.1–684.2 pg/mL; geldings: 515.0 pg/mL, CI 412.8–617.2 pg/mL). There was also no significant difference ($p = 0.6$, Cohen's $d = 0.3$) between ponies (568.6 pg/mL, CI 462.8–674.3 pg/mL) and horses (543.6 pg/mL, CI 459.3–627.8 pg/mL).

4 | DISCUSSION

Our hypothesis that feeding would affect the b/pACTH was not confirmed in this cohort of horses and ponies. The therapy with pergolide decreased the bACTH as horses in PPID group had higher bACTH than treated animals. However, the treatment with pergolide seemed to have little effect on pACTH as horses in treated-PPID group had highest results, thus, TST alone should be used with caution to adjust the dosage of pergolide in horses. Month of testing did not affect the bACTH in this study but had an impact on the pACTH with the highest results in June, July and November. The grey colour described to increase the bACTH in the autumn, did not affect the bACTH in other seasons, but had a significant impact on the pACTH. Furthermore, there is evidence, that re-testing horses within 2 weeks might decrease bACTH.

4.1 | Test performance

The bACTH can be performed at any time of the day[1] but it remains unclear whether the pACTH follows the circadian rhythm. Therefore, all the feeding protocols were applied at night, 12 h prior to testing in this study, and the test was conducted in the morning to avoid the impact of photoperiodicity.[28]

The protilerin dosage of 1 mg was used in all animals, including ponies, as it is proven to have the highest sensitivity and specificity.[29] However, the Equine Endocrinology Group recommends 0.5 mg for ponies weighing less than 250 kg.[1] Recent data show, that increasing the dosage to 2 mg or lowering it to 0.5 mg does not significantly alter the outcome and we assume that our findings would also remain similar with different dosages.[29,30]

The TST was safely performed in all horses and side effects were considered minor. Most of them occurred and disappeared within 10 min similarly to other studies,[10,11] therefore, longer observation after TST does not seem necessary. Yawning, lip smacking, flehmen response, coughing, sneezing or nose rubbing, pawing and head shaking were described in other studies[1,10,11]; however, urination, single episode of loose faeces, flatulence and trembling were not reported previously. They were possibly not considered as an adverse reaction by the other authors, or they are very rare. The mechanism of those reactions remains unclear, but a sudden increase of ACTH might potentially evoke sympathetic response leading to decreased production of tears, saliva and mucus in the respiratory system. The dry sensation in the head area might explain reactions, such as lip smacking and sneezing. However, the biological activity of ACTH from pars intermedia and subsequent increased adrenal secretion in PPID is questionable.[31] Stress accompanying the test/injection can also cause an episode of loose faeces.[32]

Care was taken to manage the samples according to the newest recommendations[33]—the blood samples were kept cool and processed to further shipping to an external laboratory within 2 h of collection. All measurements were performed by chemiluminescence immunometric assay.[34]

At least 2 weeks of washout period was provided between the tests as repeated TST led to decrease of subsequent measurements of pituitary hormones in horses tested every 3 or 7 days.[30,35] Furthermore, Kam et al.[36] reported that the repeatability of the tests 2 weeks apart was good in winter. Unfortunately, the subsequent measurements of bACTH (and to lesser extent also pACTH) were lower in this study. It can be caused by familiarisation with the examination and thus, less stress-related ACTH secretion in following tests. Nevertheless, the size effects of these findings were considered low and should not have considerable effects on the study results.

4.2 | Non-horse-related factors

4.2.1 | Feeding

The overnight feeding 12 h prior to the TST had little effect on the b/pACTH in described cohort of horses and ponies. These findings are similar to those of Restifo et al.,[11] who did not find any difference between fasted and fed horses. Nevertheless, we cannot exclude the influence of the feeding on b/pACTH due to low number of observations in this study. The discrepancies between studies might be due to different feeding protocols applied. The type of feed might have significant impact on TST and the influence of different hay type (e.g., grass, alfalfa) or concentrate (e.g., oat, corn, barley) remains to be investigated. Furthermore, the timing of grain feeding might alter the b/pACTH results, as there is evidence that long-term grain feeding in endocrinologically healthy horses increases bACTH,[9] and only short-term oat feeding was tested in this study. The special dietary management prior to testing[1] should therefore still be applied.

4.2.2 | Month

The month of testing did not influence the bACTH in the study time, which conflicts with the newest recommendations of the Equine Endocrinology Group, where higher normal values were described for June and November.[1] The low number of observations in these months might have been responsible for not detecting these differences.

The month had a significant impact on the pACTH, with the lowest results observed in April (Figure 3), similar to the findings of Miller et al.[37] The highest pACTH were obtained in July and November, what is in agreement with the newest recommendations of the Equine Endocrinology Group, that TST should no longer be performed to confirm PPID between July and December.[1] Miller et al.[37] also reported high pACTH and ΔACTH (= pACTH – bACTH) in November,

compared with August, September and October. Further studies with more observations in each month should be conducted to confirm these findings as new reference ranges might be necessary for some months, as was suggested for the bACTH in June and November.[1,19]

4.3 | Horse-related factors

4.3.1 | PPID and treatment effect

There was no significant difference in the bACTH between horses from the healthy and treated group, suggesting that pergolide can improve the bACTH and is able to reduce it to the reference range.[25,38,39] However, some studies show that this is not always the case, and the duration of therapy is also important.[40,41] The bACTH decreased only in 6/8 horses involved in our study two times (included in PPID group and re-tested in treated group). The dosage of pergolide and the corresponding clinical improvement were out of scope of this study.

Treated-PPID animals had highest pACTH (Figure 2) because of their advanced disease and earlier diagnosis compared with PPID-group. Nevertheless, there was no significant difference in the pACTH between treated and untreated horses, suggesting that pergolide has little influence on the TST results. The pACTH improved in 5/8 horses that were tested before and after therapy, but the clinical improvement was not objectively assessed. These findings are similar to the study of Durham,[42] who described the decrease of the numerical pACTH after treatment, however, most cases did not return to the normal reference range. Furthermore, Banse and McFarlane[40] also reported no significant decrease in the pACTH in horses treated with pergolide over 12 weeks. There was also no difference in the pACTH between horses receiving pergolide and placebo. Miller et al.[37] also described that horses receiving pergolide did not reach the pACTH values of healthy horses and the authors do not recommend TST to adjust the treatment of PPID. Whether the proper individual dosage of pergolide and corresponding clinical improvement decrease the pACTH into the reference range still needs to be investigated.

4.3.2 | Grey effect

Colour appears to have an effect on the bACTH with grey horses having significantly higher values in autumn.[17] The grey colour did not affect the bACTH in non-autumn months in this study, similarly to a study of Altmeyer et al.[43] Nevertheless, grey horses had significantly higher pACTH in this study. Alpha-melanocyte-stimulating hormone (α-MSH) increases the production of melanin by melanocytes and is responsible for dark skin pigmentation.[43] It is produced alongside with ACTH by melanotropes in the pituitary gland and its concentration increases in horses with PPID.[4,44] The correlations between grey colour, α-MSH, ACTH and PPID warrant further investigation as the b/pACTH measurements in grey horses in this study was limited.[24]

4.3.3 | Other effects

To improve the practical aspect of the study and reach better comparability between the groups, all animals were older than 16 years, as the prevalence of PPID in older horses is 21%.[2] There was no association between age and b/pACTH in our study, in contrast to recent literature[2,18] and the assumption, that decreasing number of dopaminergic neurons increases ACTH secretion with age.[45] This difference might be due to the narrow age range (Table 1) and the exclusion of younger animals in this study. Furthermore, Durham and Shreeve[17] described that the differences in the bACTH in older horses were most apparent between July and November, when the study was not performed.

There were no donkeys[19,26,46] or Arabians[19] in the final group, in which the increase in the bACTH is significant all over the year. There is some evidence that thrifty breeds, such as ponies, have a higher bACTH than other equids.[26,47–49] However, this finding is inconsistent,[2,49,50] and it was proved significant almost exclusively in the autumn,[17,19,48,49,51] when our study was not performed. The significantly higher pACTH was also described in Mustangs compared with Thoroughbreds in October, but not in July.[52] We did not detect any differences in the b/pACTH between ponies and other breeds. The discrepancies between studies might be caused by collecting many pony breeds together. The significantly higher bACTH was described in Shetland, mini-Shetland and Welsh A ponies compared with other pony breeds.[17,19,51] Further studies with a higher number of certain pony breeds in each month are necessary.

4.4 | Limitations

The biggest limitation of our study was the small number of horses. The power of the test was 95% and calculated to determine the minimal number of horses in each group to prove the influence of feeding on the bACTH. The power of the test for findings in the linear mixed model was not calculated and might be much lower for findings with low number of observations. However, the effect sizes were calculated to better assess the magnitude of differences.

The second biggest limitation proved to be the decrease of the b/pACTH in subsequent tests, what could have affected our results. Nevertheless, the size effect was considered low.

There was also a discrepancy between the categorisation of few horses into groups created based on our inclusion criteria (bACTH, clinical signs and treatment) and the results of pACTH. Two horses from the healthy group had positive results of at least two TSTs and three horses from the untreated PPID group had negative results of at least two TSTs. Inclusion of horses with questionable bACTH results might be the reason for this false categorisation. Nevertheless, horses from the 'grey zone' are the target group for TST,[1,5–7] thus, they were included in our study. Furthermore, we used the reference ranges for the b/pACTH interpretation suggested by Schott et al.[22] from 2019 and new values are recommended since 2021 by Hart et al.[1] Applying the new criteria retrospectively in our study considerably increased the number of animals with equivocal bACTH (12 results were

changed from unlikely to equivocal, one from likely to equivocal and three from equivocal to likely), however, the categorisation of horses did not change in any case.

A minimal delay in blood collection after TST can lead to significant differences in the ACTH results.[28] We aimed at blood collection exactly 10 min after the injection, but there was a one-minute delay in two cases, what could lead to falsely low ACTH measurements in those horses.[28] The use of a catheter in difficult horses should therefore be considered.[10] Furthermore, the blood collection after 30 min is reported to be less affected by the timing of the sample collection.[8,28,53]

Finally, the acute stress seen in a few horses might have also influenced our results,[16,54] especially in horses that were isolated from the herd for fasting.

5 | CONCLUSION

There was little evidence that feeding with oat 2 h before or fasting 12 h prior to the b/pACTH measurements have impact on both results. Horses with PPID (regardless the treatment) and grey coloured were shown to have highest pACTH in this cohort. Animals in the treated-PPID group had a lower bACTH than untreated horses, what suggests an influence of pergolide on bACTH. However, the drug seems to have little effect on pACTH as horses in the treated-PPID group had the highest results. Despite the significance and at least moderate effect size of these findings, they warrant further examinations on a larger group of animals.

AUTHOR CONTRIBUTIONS

Karolina Drozdzewska participated in the study design, collected the data, participated in the data analysis and interpretation, prepared the original manuscript and had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of data analysis. Judith Winter, Ann Kristin Barton and Heidrun Gehlen participated in the study design and the critical article revision. Roswitha Merle participated in the data analysis, interpretation and the critical article revision. All authors gave their final approval of the manuscript.

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The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request: Open sharing exemption granted by editor for this clinical report.

ETHICAL ANIMAL RESEARCH

The experiment was approved by State Office for Health and Social Affairs in Berlin, Germany (G 0237/19).

INFORMED CONSENT

Owners gave consent for their animals' inclusion in the study.

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5. Diskussion

5.1 Tumormarker in der Pferdemedizin

In dem ersten Teil der Arbeit wurde ein Review der Literatur über Tumormarker beim Pferd, inklusive ACTH durchgeführt. Drei Gruppen von Tumormarkern sind beschrieben: Hormone (z.B. ACTH, Alpha-Fetoprotein), Enzyme (z.B. Thymidinkinase 1, Alkalische Phosphatase) und Antikörper (z.B. monoklonale Gammopathie, autologe Antikörper gegen Erythrozyten oder Thrombozyten). Leider ist keine der beschriebenen Substanzen ein idealer Biomarker und ein allgemeines Screening auf Neoplasien bei Pferden wird nicht empfohlen. Die Ergebnisse sollten zusammen mit der Anamnese, dem Signalement, verdächtigen klinischen Symptomen oder Laborbefunden (z.B. paraneoplastische Syndrome) interpretiert werden. Viele Faktoren erhöhen die Konzentration oder Aktivität der Tumormarker. Die häufigsten sind junges Alter, Trächtigkeit, Laktation, Entzündung, Infektion oder schwere Erkrankung. Die Interpretation der Ergebnisse gleichzeitig mit Entzündungsmarkern kann helfen, falsch-positive Ergebnisse zu vermeiden und in Tumorprofilen sind diese in der Regel integriert. Bei entzündlichen Erkrankungen sollen sich die Werte normalisieren, sobald die Entzündung abklingt, weshalb wiederholte Messungen empfohlen werden. Auf der anderen Seite schreit das Tumorwachstum in der Regel fort. Daraus resultierend bleiben Folgemessungen erhöht oder verzeichnen einen Anstieg. Es sind weitere Studien erforderlich, um die Sensitivität und Spezifität von Tumormarkern in der Pferdemedizin zu spezifizieren.

5.2 ACTH als Tumormarker

In der Literatur wurden mehrere Faktoren beschrieben, die einen Einfluss auf bACTH und teilweise auf pACTH als Tumormarker für das Hypophysenadenom oder -karzinom haben. Zu den wichtigsten gehören Jahreszeit (Copas und Durham 2012), Stress und Krankheit (Stewart et al. 2019; Haffner et al. 2020), Alter (Mcfarlane 2007; Durham und Shreeve 2017), primitive Rasse (Fredrick und Mcfarlane 2014; Durham und Shreeve 2017; Bamford et al. 2020; Potier und Durham 2020), weibliches Geschlecht (Durham und Shreeve 2017) und Schimmelfarbe (Durham und Shreeve 2017). Des Weiteren gab es eine Diskrepanz zwischen Studien bezüglich des Einflusses der Fütterung auf b/pACTH (Diez De Castro et al. 2014; Restifo et al. 2016; Jacob et al. 2018).

Die zweite Publikation ist eine Studie, die bekannte Einflussfaktoren auf b/pACTH überprüft. Die in der Studie aufgenommenen Tiere waren klinisch gesund und älter als 16 Jahre, um den Einfluss von akuter Krankheit und Alter zu minimieren. Des Weiteren wurde die Studie nicht im Spätsommer und Herbst durchgeführt. Es wurden alle Rassen (inklusive Ponys), beide Geschlechter, verschiedene Fellfarben, endokrinologisch gesunde und an PPID erkrankte

Pferde, mit und ohne Behandlung, aufgenommen und anschließend verglichen. Um den Einfluss fehlerhafter Probenbearbeitung und unterschiedlicher Labormessmethoden (Mcgilvray et al. 2020; Stewart et al. 2020) zu vermeiden, wurde besonders drauf geachtet, dass die Blutproben ständig gekühlt waren, innerhalb von zwei Stunden nach der Entnahme verarbeitet und durch dasselbe externe Labor innerhalb von 24 Stunden gemessen wurden (Hart et al. 2021).

5.3 Durchführung des TRH-Stimulationstests

Der TRH-Stimulationstest wurde bei allen Pferden sicher durchgeführt und Nebenwirkungen wurden als geringfügig eingestuft. Die meisten von ihnen traten auf und verschwanden innerhalb von zehn Minuten ähnlich wie in anderen Studien (Diez De Castro et al. 2014; Restifo et al. 2016), daher scheint eine längere Beobachtung der Patienten nach dem TRH-Stimulationstest nicht notwendig zu sein. Gähnen, Lippen lecken, Flehmen, Husten, Niesen oder Nase reiben, Scharren und Kopfschütteln wurden in anderen Studien beschrieben (Diez De Castro et al. 2014; Restifo et al. 2016; Hart et al. 2021). Von Harnabsatz, weichem Kotabsatz, Flatulenzen und Zittern wurde jedoch zuvor nicht berichtet. Stress, der die Injektion begleitet, kann auch eine Episode von weichem Kot verursachen (Kienzle et al. 2016). Der Mechanismus anderer Reaktionen bleibt unklar, aber ein plötzlicher Anstieg von ACTH könnte möglicherweise eine sympathische Reaktion hervorrufen, die zu einem trockenen Gefühl im Kopfbereich führte, was das Lippenlecken und Niesen erklären könnte. Ein Zusammenhang zwischen der biologischen Aktivität von ACTH aus der Pars intermedia und der anschließend erhöhten Nebennierenfunktion bei PPID ist jedoch fraglich (Boujon et al. 1993).

5.4. Einflussfaktoren auf b/pACTH

Unsere Hypothese, dass die Fütterung das b/pACTH beeinflussen würde, wurde in dieser Kohorte von Pferden und Ponys nicht bestätigt. Diese Ergebnisse ähneln denen von Restifo et al. (2016), die keinen Unterschied zwischen gefasteten und gefütterten Pferden fanden. Dennoch können wir den Einfluss der Fütterung auf das b/pACTH aufgrund der geringen Probandenzahl in dieser Studie nicht ausschließen. Die Diskrepanzen zwischen vergangenen Studien könnten auf unterschiedliche Fütterungsprotokolle zurückzuführen sein. Die Art des Futters könnte einen signifikanten Einfluss auf den TRH-Stimulationstest haben und der Einfluss verschiedener Heusorten (z.B. Gras, Luzerne) oder Kraftfutter (z.B. Hafer, Mais, Gerste) bleibt zu untersuchen. Außerdem könnte der Zeitpunkt der Getreidefütterung die b/pACTH-Ergebnisse verändern, da es Hinweise gibt, dass eine langfristige Getreidefütterung bei endokrinologisch gesunden Pferden das bACTH erhöht (Jacob et al. 2018) und in dieser Studie wurde nur eine kurzfristige Haferfütterung getestet. Die spezielle

Fütterungsempfehlungen vor dem Testen sollten daher weiterhin angewendet werden (Hart et al. 2021; Menzies-Gow et al. 2023).

Der Monat hatte keinen Einfluss auf das bACTH in dieser Studie, was im Widerspruch zu den neuesten Empfehlungen der Equine Endocrinology Group (Hart et al. 2021) steht, wo höhere Normwerte für Juni und November beschrieben wurden. Die geringe Anzahl von Untersuchungen in diesen Monaten könnte dafür verantwortlich gewesen sein, dass diese Unterschiede nicht erkannt wurden. Der Monat hatte jedoch einen signifikanten Einfluss auf das pACTH, mit den höchsten Werten im Juli und November, was die aktuelle Literaturempfehlung, den TRH-Stimulationstest nicht mehr zwischen Juli und Dezember durchzuführen, unterstützt (Hart et al. 2021; Miller et al. 2021). Um diese Ergebnisse zu bestätigen, sollten weitere Studien mit höheren Probandenzahlen in jedem Monat durchgeführt werden, da für einige Monate neue Referenzbereiche notwendig sein könnten.

Es ist bewiesen, dass die Therapie mit Pergolid das bACTH senkt (Perkins et al. 2002; Mcfarlane et al. 2017; Kirkwood et al. 2022) und auch in unserer Studie hatten Pferde in der PPID-Gruppe einen signifikant höheren Wert als behandelte Tiere. Die Behandlung mit Pergolid schien jedoch wenig Einfluss auf das pACTH zu haben, ähnlich wie in anderen Studien (Banse und Mcfarlane 2017; Miller et al. 2021; Durham 2022). Der TRH-Stimulationstest ist daher nach aktuellem Kenntnisstand nicht geeignet, um die Dosierung von Pergolid bei PPID-erkrankten Pferden anzupassen (Menzies-Gow et al. 2023). Es muss noch geklärt werden, ob eine klinische Verbesserung und eine angepasste Pergolid-Dosierung zu einer Normalisierung des pACTH führen kann (Durham 2022).

Schimmel weisen höheres bACTH im Herbst auf (Durham und Shreeve 2017), aber die Fellfarbe hatte keinen Einfluss auf das bACTH in anderen Jahreszeiten in unserer Studie, ähnlich wie von Altmeyer et al. (1984) beschrieben. Es war jedoch ein signifikanter Einfluss auf das pACTH festzustellen. Das α-MSH wird zusammen mit ACTH von Melanotropen in der Hypophyse produziert und seine Konzentration steigt bei Pferden mit PPID (Beech et al. 2011b; Mc Gowan et al. 2013). Die Zusammenhänge zwischen grauer Farbe, α-MSH, ACTH und PPID bedürfen weiterer Untersuchungen, da die Anzahl der Schimmel in dieser Studie begrenzt war.

Es gab keinen biologisch relevanten Zusammenhang zwischen Alter und b/pACTH in unserer Studie, im Gegensatz zur aktuellen Literatur (Mcfarlane 2007; Mcgowan et al. 2013; Mcfarlane und Maxwell 2017). Dieser Unterschied könnte auf die geringe Altersspanne und den Ausschluss jüngerer Tiere in dieser Studie zurückzuführen sein. Außerdem wurde der größte Unterschied im bACTH bei älteren Pferden zwischen Juli und November beschrieben (Durham und Shreeve 2017), ein Zeitraum, in dem unsere Studie nicht durchgeführt wurde.

Es gibt einige Hinweise, dass primitive Rassen und Ponys ein höheres bACTH als andere Equiden haben (Mcfarlane et al. 2011; Bamford et al. 2020; Humphreys et al. 2022), andere

Studien beweisen keinen Unterschied (Mcgowan et al. 2013) oder signifikanten Unterschied ausschließlich im Herbst (Durham und Shreeve 2017; Bamford et al. 2020; Durham et al. 2022). Wir konnten keine Erhöhung von b/pACTH bei Ponys feststellen, aber unsere Studie umfasste mehrere Ponyrassen. Das signifikant höhere bACTH wurde bei Shetland-, Mini-Shetland- und Welsh-A-Ponys im Vergleich zu anderen Ponyrassen beschrieben (Durham und Shreeve 2017; Durham et al. 2022). Weitere Studien mit einer höheren Anzahl bestimmter Ponyrassen in jedem Monat sind notwendig.

5.5 Limitationen

Die größte Limitation unserer Studie war die geringe Anzahl von Tieren. Die Teststärke betrug 95% und wurde berechnet, um die minimale Anzahl von Pferden in jeder Gruppe zu bestimmen, die erforderlich war, um den Einfluss der Fütterung auf das bACTH zu evaluieren. Die Teststärke für die restlichen Analysen wurde nicht berechnet und könnte für Befunde mit geringer Anzahl von Beobachtungen viel niedriger sein.

Als zweitgrößte Einschränkung erwies sich die Abnahme der bACTH-Konzentration (und in geringerem Maße auch der pACTH-Konzentration) in den nachfolgenden Messungen bei jedem Pferd, obwohl in der Literatur eine als sinnvoll beschriebene Auswaschphase von mindestens zwei Wochen zwischen den Tests vorgesehen wurde (Mccann et al. 1992; Schorn et al. 2020) Kam et al. (2021). Dies könnte durch eine Gewöhnung an die Untersuchung und somit eine geringere stressbedingte ACTH-Sekretion in den folgenden Tests, verursacht worden sein. Die Größeneffekte dieser Befunde wurden jedoch als gering eingestuft und sollten keine erheblichen Auswirkungen auf die Studienergebnisse haben.

Des Weiteren gab es bei wenigen Pferden eine Diskrepanz zwischen der Kategorisierung in Gruppen, die basierend auf unseren Einschlusskriterien erstellt wurden, und den Ergebnissen des pACTH. Die Einbeziehung von Pferden mit grenzwertigen bACTH könnte der Grund für den Fehler sein. Dennoch sind die Pferde aus der “grauen Zone” die Zielgruppe für den TRH-Stimulationstest (Beech et al. 2007;Gough et al. 2019), daher wurden sie in unsere Studie einbezogen.

Eine minimale Verzögerung bei der Blutentnahme nach dem TRH-Stimulationstest kann zu signifikanten Unterschieden in den ACTH-Ergebnissen führen (Thane et al. 2022). Wir strebten eine Blutentnahme exakt zehn Minuten nach der Injektion an, aber es gab eine einminütige Verzögerung in zwei Fällen, was zu falsch niedrigen pACTH-Werten führen könnte. Die Verwendung eines Katheters bei schwierigen Pferden sollte daher in Betracht gezogen werden (Diez De Castro et al. 2014). Schließlich könnte der akute Stress, der bei einigen Pferden beobachtet wurde, auch unsere Ergebnisse beeinflusst haben (Haffner et al. 2020).

6. Zusammenfassung

Einfluss der Fütterung auf den ACTH Basalwert und den TRH Stimulationstest bei endokrinologisch gesunden und an Pituitary Pars Intermedia Dysfunction erkrankten Pferden.

Einige Tumormarker wurden in der Pferdemedizin beschrieben, keiner davon ist jedoch ideal. Die Substanzen kommen inkonsistent im Blut vor und viele Faktoren und Krankheiten können ihre Konzentration oder Aktivität steigern, z.B. Entzündung, schwerwiegende Erkrankung und Alter. Infolgedessen wird ein Screening für innere Neoplasien bei Pferden nicht empfohlen, weil es zu falsch positiven Ergebnissen führen kann. Die Biomarker sollen immer zusammen mit dem Vorbericht des Patienten, seiner klinischen Untersuchung und klinisch-pathologischen Ergebnissen evaluiert werden, die verdächtig für das Vorliegen eines bestimmten Tumors sind (z.B. ein paraneoplastisches Syndrom). Eine gleichzeitige Interpretation von Entzündungsmarkern oder wiederholte Messungen können die Genauigkeit des Tumormarkers verbessern.

Adrenocorticotropin (ACTH) ist ein Hormon, das weltweit als Tumormarker für ein Hypophysenadenom oder -adenokarzinom bei Pferden mit Pituitary Pars Intermedia Dysfunktion (PPID) eingesetzt wird. Es gibt zahlreiche Faktoren, die die basale ACTH-Konzentration (bACTH) beeinflussen können, inkl. Stress, schwerwiegende Krankheit, Alter und Monat. Des Weiteren gibt es Faktoren, die bACTH-Konzentration nur im Herbst beeinflussen, z.B. Schimmel-Farbe, Pony-Rasse und weibliches Geschlecht. Die Sensitivität des Tests kann weiter erhöht werden durch eine Bestimmung der ACTH-Konzentration nach einer Stimulation mit Thyrothropin-Releasing Hormon (pACTH), aber auch das Ergebnis kann durch Monat und schwerwiegende Krankheit verändert werden. Die Auswirkung anderer Faktoren auf pACTH ist bis jetzt spärlich beschrieben.

Beide Untersuchungen sind nach Heu-Fütterung empfohlen, aber es gibt eine Diskrepanz in der Literatur über die Auswirkung von Kraftfutter und Fasten. Das Ziel unserer Studie war, den Einfluss verschiedener Futter-Protokollen, Schimmel-Farbe, weiblichem Geschlecht, Pony-Rasse, Monat, Alter und Body Condition Score (BCS) auf die b/pACTH-Konzentration bei Pferden und Ponys zu ermitteln.

Einundsechzig Tiere älter als 16 Jahre wurden in drei Gruppen basierend auf klinischen Symptomen, Behandlung und bACTH verteilt: gesund = 20, PPID = 21, PPID behandelt mit Pergolid = 20. Die Studie hatte ein Crossover-Design und fand zwischen Mitte November und Mitte Juli statt. Die Messung von b/pACTH wurde drei Mal bei jedem Tier nach unterschiedlichen Futter-Protokollen (Fasten, nur Heu, Heu + Kraftfutter) durchgeführt und die

Reihenfolge wurde stratifiziert randomisiert. Die Blutentnahme erfolgte vor und exakt zehn Minuten nach einer intravenösen Injektion von 1 mg Protirelin.

Der TRH-Stimulationstest wurde erfolgreich bei allen Pferden durchgeführt. Die Nebenwirkungen waren geringfügig und dazu gehörten vor allem Schnaufen, Lippenlecken, Juckreiz, Kotabsatz und Flatulenzen. Die Hypothese, dass die Fütterung einen Einfluss auf b/pACTH hat, wurde in dieser Studie nicht bestätigt und es gab keine Unterschiede in b/pACTH zwischen den verschiedenen Futter-Protokollen (Friedman-Test). Nichtsdestotrotz sollten das Anbieten unterschiedlicher Typen an Kraft- und Rauffutter sowie unterschiedliche Fütterungszeiten weiter untersucht werden.

Im Mixed Modell wurde das bACTH durch die Gruppe beeinflusst und die niedrigsten Werte wurden bei gesunden und die höchsten bei nicht-behandelten PPID-Pferden gemessen, was bestätigt, dass Pergolid das bACTH reduzieren kann. Es gab Hinweise, dass eine wiederholte Messung innerhalb von zwei Wochen zu einer Reduktion des bACTH führen kann, was auf eine Gewöhnung des Tieres an die Untersuchung zurückzuführen ist.

Das pACTH wurde durch die Gruppe, Monat und Schimmel-Farbe beeinflusst. Pergolid hatte wenig Effekt auf pACTH und die Ergebnisse waren am höchsten in der behandelten PPID-Gruppe, welche sich nicht signifikant von denen der nicht-behandelten PPID-Gruppe unterschied. Das pACTH war am höchsten im Juli und im November und basierend auf den neuen Empfehlungen der Equine Endocrinology Group (2021), sollte der TRH-Stimulationstest nicht mehr in diesen Monaten durchgeführt werden. Alternativ sollten neue Referenzwerte entwickelt werden. Interessanterweise wiesen Schimmel signifikant höheres pACTH im Vergleich zu anderen Farben auf.

Die Resultate deuten drauf hin, dass die Fütterung über Nacht wenig Effekt auf das b/pACTH hat. Es gibt Evidenz, dass eine Therapie mit Pergolid das bACTH reduzieren kann, aber es hat wenig Effekt auf pACTH. Die beschriebenen Ergebnisse rechtfertigen weitere Studien in einer größeren Tiergruppe, mit mehreren Untersuchungen pro Monat und einer größeren Schimmel-Population. Des Weiteren sollte weiter geforscht werden, ob eine klinische Verbesserung und eine angepasste Pergolid-Dosierung zu einer Normalisierung des pACTH bei Pferden mit PPID führen sollte. Eine zuverlässige PPID-Diagnose kann nur gestellt werden, wenn alle Einflussfaktoren auf b/pACTH erkannt werden.

7. Summary

Influence of feeding on basal ACTH concentration and TRH stimulation test in endocrinologically healthy and with Pituitary Pars Intermedia Dysfunction affected horses and ponies.

There are several tumour markers described in equine medicine, however, none of them is ideal. The presence of this substances in blood is inconsistent and many factors and diseases might increase their activity or concentration, with inflammation, critical illness and age being the most common examples. Consequently, it is not recommended to perform a screening for internal neoplasia in horses as it can lead to false positive results. The biomarkers should be evaluated together with the patient history, physical examination and clinicopathological findings, which should be suspicious for the certain internal tumour (e.g., presence of paraneoplastic syndrome). Simultaneous interpretation of inflammatory markers or repeated measurements can further improve the accuracy of tumour marker.

Adrenocorticotropin (ACTH) is a hormone, which is widely used as a tumour marker of pituitary adenoma or adenocarcinoma in horses with Pituitary Pars Intermedia Dysfunction (PPID). There are plenty of factors influencing the basal concentration of ACTH (bACTH), including stress, severe disease, age and month. Furthermore, some factors affect the bACTH concentration only in autumn, e.g., grey colour, pony breed and female sex. The sensitivity of the test can be further increased by measuring the ACTH concentration after stimulation with thyrotropin-releasing hormone (pACTH), but also this measurement is influenced by the month and severe disease. The impact of other factors on pACTH is sparsely described so far.

Both tests are recommended after feeding hay only but there are discrepancies in recent literature regarding the influence of grain and fasting. The goal of our study was to investigate whether different feeding protocols, grey colour, female sex, pony breed, month, age and Body Condition Score (BCS) affect the b/pACTH in horses and ponies.

Sixty-one animals aged > 16 years were divided into three groups based on clinical signs, treatment and bACTH: healthy = 20, PPID = 21, PPID treated with pergolide = 20.

The study had a crossover design and it was conducted between mid-November to mid-July. The measurement of b/pACTH was performed three times in each animal after different feeding protocols (fasting, hay only, hay + grain) and their order was stratified randomised. Blood sample was taken before and exactly ten minutes after intravenous injection of 1 mg of protirelin.

TRH stimulation test was successfully performed in all horses. Side effects were considered minor and included mainly sneezing, lip smacking, itching, passing faeces and flatulence. The hypothesis that feeding affects the b/pACTH was not confirmed in this study as there was no difference in b/pACTH after applying different feeding protocols (Friedman´s test). However, offering different types of grain or roughage and different timing of feeding should be further investigated.

In the linear mixed model, the bACTH was influenced by a group with lowest results in healthy animals and highest in untreated-PPID horses, what confirms that therapy with pergolide can reduce bACTH. There was evidence that re-testing animals within two weeks might decrease subsequent bACTH, what might be due to the habituation with the examination.

The pACTH was affected by group, month and grey colour. Pergolide proved to have little effect on pACTH as the measurements were the highest in treated-group and not significantly different from untreated-PPID group. The pACTH was highest in July and November and according to the new recommendation of Equine Endocrinology Group (2021), the TRH stimulation test should not be performed in these months anymore. Alternatively, new reference ranges should be considered. Interestingly grey horses showed significantly higher pACTH than other coat colours.

The results suggest that overnight feeding has little effect on b/pACTH. There is evidence that treatment with pergolide can reduce bACTH but has little effect on pACTH.

Described findings warrant further investigation on a larger group of animals with more observations pro month and higher percentage of grey horses. Furthermore, it remains to be investigated whether corresponding clinical improvements and adjusted pergolide dosage should be accompanied by decrease of pACTH to the reference range in horses with PPID. The reliable diagnosis of PPID can be made only after recognition of all influencing factors on b/pACTH.

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Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

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