UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

CONTRIBUTION TO THE EVALUATION OF THE BENEFITS OF PERGOLIDE IN THE TREATMENT OF EQUINE PITUITARY PARS INTERMEDIA DYSFUNCTION (PPID): REDUCTION OF HELMINTH FAECAL EGG COUNTS (HFEC) AND INCREASED OWNER AWARENESS

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Garcia de Andrade Pimenta Tilley

2023

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CONTRIBUIÇÃO PARA A AVALIAÇÃO DOS BENEFÍCIOS DA PERGOLIDA NO TRATAMENTO DA DISFUNÇÃO DA PARS INTERMEDIA DA HIPÓFISE EQUINA (DPIP): REDUÇÃO DAS CONTAGENS DE OVOS DE HELMINTES NAS FEZES E AUMENTO DA CONSCIENCIALIZAÇÃO DOS PROPRIETÁRIOS

Resumo

A expectativa de vida dos equinos tem vindo a aumentar e a disfunção da *pars intermedia* da pituitária (DPIP) é a doença endócrina mais comum em cavalos mais velhos. O tratamento padrão é baseado na administração de pergolida, no entanto a DPIP é frequentemente subdiagnosticada, já que os proprietários atribuem os seus sinais clínicos apenas ao envelhecimento. Devido à imunossupressão, esses cavalos podem ter infeções e contagens de ovos de helmintes nas fezes mais altas e podem requerer um programa de controlo de parasitas mais agressivo.

No presente estudo, quinze cavalos foram escolhidos com base em exames clínicos e concentração de hormona adenocorticotrófica (ACTH). Dez deles foram tratados com pergolida (grupo de tratamento) e cinco não receberam medicação (grupo de controlo), de maneira a avaliar o efeito da pergolida oral na redução da contagem fecal de ovos de helmintes e no tempo de reaparecimento de ovos ao longo de 6 meses. Inicialmente, todos os cavalos foram desparasitados e as contagens foram realizadas mensalmente ao longo do período do estudo. Realizou-se ainda um questionário, que foi aplicado nos dias 1 e 180 do estudo, com o objetivo avaliar a consciencialização sobre a doença e registou-se também a adesão ao tratamento por parte dos proprietários.

Embora não seja estatisticamente significativo, ao longo dos 5 períodos de avaliação houve maiores percentagens de 0 ovos por grama de fezes (OPG) e menos casos de valores clinicamente relevantes de OPG no grupo de tratamento quando comparados com o grupo de controlo. Relativamente à medição de condição corporal, esta mostrou diferenças estatisticamente significativas entre os grupos, sendo superior no grupo de tratamento. Cavalos sob tratamento com pergolida mantiveram melhores valores quanto ao peso, embora sem significância estatística.

A influência dos benefícios da pergolida na qualidade de vida de cavalos com DPIP foi demonstrada, contribuindo para aumentar a consciencialização dos proprietários sobre a doença, tendo havido adesão total ao tratamento por parte dos proprietários dos animais em estudo.

Palavras-chave: DPIP, Pergolida, Contagem de ovos fecais, equino, ACTH

CONTRIBUTION TO THE EVALUATION OF THE BENEFITS OF PERGOLIDE IN THE TREATMENT OF EQUINE PITUITARY PARS INTERMEDIA DYSFUNCTION (PPID): REDUCTION OF HELMINTH FAECAL EGG COUNTS (HFEC) AND INCREASED OWNER AWARENESS

Abstract

Equine life expectancy is increasing and PPID is the most common endocrine disorder of older equids. The standard treatment is pergolide but PPID is often underdiagnosed as owners attribute its clinical manifestations to old age alone. Due to immunosuppression, these horses may have infections and higher helminth faecal egg counts (hFEC) and can require a more aggressive parasite control program.

In the present study, fifteen horses were enrolled based on clinical examination and ACTH concentration. Ten of these were treated with pergolide (treatment group) and five received no medication (control group), in order to evaluate the effect of oral pergolide in the reduction of hFEC and time to egg reappearance over 6 months. Initially, all horses were de-wormed and hFEC were carried out monthly over the study period. A questionnaire was also conducted, which was applied on days 1 and 180 of the study, with the aim of assessing awareness of the disease, and the owners' adherence to the treatment was recorded as well.

Although not statistically significant, there were higher percentages of 0 EPG and fewer cases of clinically relevant values of EPG over the 5 evaluation periods in the treatment group when compared to the control group. The body condition score showed statistically significant differences between groups, being higher in the treatment group. Horses under pergolide treatment were better weight keepers, although without statistical significance.

The influence of pergolide benefits in the quality of life of horses with PPID was reinforced, contributing to increase owner awareness of the disease. Owner adherence was 100%.

Keywords: PPID, Pergolide, Faecal egg count, horse, ACTH

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Resumo alargado

Devido ao aumento na expectativa de vida e consequente envelhecimento da população equina, a medicina geriátrica está cada vez mais a tornar-se uma parte importante na medicina veterinária de equinos. A disfunção da pars intermedia da pituitária é a doença endócrina mais comum em equinos mais velhos (especialmente ≥ 15 anos de idade), o que a torna consideravelmente relevante na prática clínica (Ireland e McGowan 2018; Fortin et al. 2021; Kirkwood et al. 2022).

A DPIP é uma doença neurodegenerativa de evolução crónica e progressiva ao longo da vida (van Proosdij e Frietman 2022). A perda de resposta inibitória dopaminérgica para a pars intermedia da pituitária resulta em concentrações plasmáticas elevadas de múltiplos péptidos derivados da POMC, incluindo a hormona adrenocorticotrópica, hormona estimulante de melanócitos alfa, β-endorfina e péptido intermediário semelhante ao corticotrofina (Ireland e McGowan 2018; Horn et al. 2019; Tatum et al. 2021).

Existem vários testes laboratoriais endócrinos que podem ajudar no diagnóstico desta doença (Tatum et al. 2021). Os testes basais medem as concentrações plasmáticas de ACTH ou α-MSH e os testes dinâmicos incluem o teste de supressão com dexametasona durante a noite e o teste de estimulação com hormona libertadora de tireotropina. O teste de supressão com dexametasona durante a noite apresenta sensibilidade reduzida, especialmente em casos de doença precoce ou leve, principalmente durante o outono. O teste de escolha para o diagnóstico de DPIP é a concentração basal endógena de ACTH (Secombe et al. 2018; Durham et al. 2020). Os estudos disponíveis demonstram que a medição de ACTH basal é altamente específica e possui boa sensibilidade para o diagnóstico de DPIP em cavalos e póneis com sinais clínicos consistentes com a doença (Tatum et al. 2021).

O tratamento preferencial para DPIP é o mesilato de pergolida, um agonista de receptor de dopamina que leva à regulação negativa da produção de péptidos derivados da POMC (Jacobson et al. 2014; Meyer et al. 2022).

Os sinais clínicos de DPIP incluem hipertricose e queda anormal do pêlo, laminite, atrofia muscular epaxial ou atrofia muscular, perda de peso, distribuição anormal de gordura, polidipsia, poliúria, infecções bacterianas, parasitismo intestinal e letargia ou depressão (Ireland e McGowan 2018; Fortin et al. 2021; Tatum et al. 2021). Portanto, cavalos com DPIP tendem a ter contagens mais altas de ovos de helmintes nas fezes e podem precisar

de um programa de prevenção parasitário mais agressivo, levando a uma maior dificuldade em manter a sua condição corporal e a um custo mais alto (McFarlane et al. 2010; Horn et al. 2019). Existe então uma necessidade imperativa de educar os proprietários para identificar sinais clínicos de DPIP, uma vez que estes mesmos sinais clínicos são muitas vezes confundidos com a idade avançada apenas.

Uma vez que não há muitos estudos que avaliem o efeito da pergolida na imunossupressão (Kirkwood et al. 2022) e os seus efeitos numa possível diminuição nas contagens de ovos fecais de helmintes (hFEC), este estudo teve como objectivo avaliar o efeito da administração oral de pergolida na redução do hFEC e no tempo de reaparecimento de ovos, em comparação com a ausência de tratamento, ao longo de um período de 6 meses, em 15 cavalos com ≥ 15 anos de idade diagnosticados com DPIP em meses de não outona (ACTH plasmático > 29 µg/ml). Além disso, realizou-se também um questionário que apresentava os principais sinais clínicos desta doença com o intuito de educar os proprietários destes animais a reconhecer este sinais e identificar potenciais alterações que possam indicar o desenvolvimento desta doença.

Os cavalos foram incluídos neste estudo com base em uma suspeita clínica de DPIP, associada a um aumento no ACTH plasmático acima do intervalo de referência, que foi considerado um meio confiável de diagnóstico em estudos anteriores (McFarlane et al. 2010; Christen et al. 2018). Os resultados clínicos incluíram a avaliação da pressão sanguínea, peso, condição corporal e acumulação adiposa no pescoço, enquanto o resultado laboratorial foi a contagem de ovos fecais de helmintes (hFEC). Além disso, a percepção dos proprietários sobre os sinais clínicos desta doença foi avaliada antes e após o tratamento.

O *score* de condição corporal mostrou diferenças estatisticamente significativas entre os grupos, enquanto o *score* de acumulação adiposa no pescoço, apesar de mostrar algumas diferenças, não pôde ser considerado estatisticamente relevante. Outros estudos não mostraram diferenças nestes parâmetros quando comparando os grupos de tratamento e controlo (Banse et al. 2021; Miller et al. 2021). Em relação às mudanças de peso durante o período de teste, ambos os grupos mostraram perda significativa de peso. No entanto, mesmo havendo uma perda de peso significativa nos cavalos do grupo de tratamento na fase final do estudo (dias 90 e 180) em comparação com o dia 60, esses cavalos mantiveram sempre o peso acima dos cavalos do grupo de controlo a partir do dia 60. Além disso, a diminuição média de peso do dia 90 ao dia 180 não foi tão acentuada quanto nos cavalos do grupo de controlo, sugerindo melhor manutenção de peso pelos cavalos sob tratamento com pergolida. Por outro lado, alguns estudos anteriores relataram perda de peso nos cavalos tratados com pergolida, enquanto os cavalos não tratados mostraram manutenção ou mesmo ganho de peso (Horn et al. 2019; Banse et al. 2021). Enquanto isso, outros autores relataram que as mudanças no peso corporal não foram significativas entre

os grupos, mostrando alguma variabilidade nos resultados obtidos para este parâmetro (Miller et al. 2021).

Quanto à medição da pressão arterial, não houve diferenças estatisticamente significativas entre o dia 1 e o dia 180 para os valores sistólicos e diastólicos em ambos os grupos.

O padrão de variação circanual do ACTH mostrou ser notavelmente semelhante em cavalos com DPIP e sem DPIP, sugerindo que a regulação do fotoperíodo pela glândula pituitária parece ser preservada quando os cavalos têm essa doença (Copas and Durham 2012; Humphreys et al. 2022). Além disso, valores de referência circanuais para o ACTH foram publicados e, dado que a maior diferença nos níveis de ACTH entre cavalos saudáveis e os com DPIP ocorre entre agosto e outubro, sugere-se que, aplicando os intervalos de referência circanuais, este período pode ser o mais apropriado para testar a DPIP (Copas and Durham 2012; Durham et al. 2020). O presente estudo foi realizado entre dezembro e julho, quando os níveis de ACTH diferem menos entre os dois grupos, o que pode explicar porque ambos os grupos tiveram diminuição dos valores de ACTH.

Os estudos existentes sobre DPIP e hFEC compararam cavalos com a doença e controlos saudáveis (McFarlane et al. 2010; Christen et al. 2018). Neste estudo, comparámos cavalos com DPIP com ou sem tratamento com pergolida. A hipótese em estudo seria que cavalos com DPIP tratados com pergolida têm menor quantidade de ovos nas fezes (medição em OPG) e maior tempo até ao reaparecimento de ovos. Em relação à contagem de ovos nas fezes, usando as técnicas de McMaster e Mini-Flotac, concluiu-se que não houve diferenças significativas nas OPG de base entre os grupos. No entanto, com a técnica de McMaster, em geral, houve percentagens mais altas de 0 EPG e menos casos de valores clinicamente relevantes de OPG ao longo dos 5 períodos de avaliação no grupo de tratamento em comparação com o grupo de controlo. Além disso, os cavalos do grupo de tratamento mantiveram hFEC < 200 ovos/grama durante o estudo, e o grupo de controlo teve 1 animal que manteve hFEC quase sempre acima dos valores aceitáveis. Apesar de as diferenças entre os grupos não serem estatisticamente significativas, possivelmente devido ao pequeno tamanho da população envolvida no presente estudo, o tratamento com pergolida parece ter alguma influência indireta no controlo de parasitas.

Da análise do questionário, a informação mais relevante a destacar é o facto de os proprietários não terem conhecimento de alguns dos sinais clínicos da DPIP, pois associam-nos apenas à idade avançada dos animais. Na verdade, no questionário final, alguns proprietários mencionaram algumas melhorias nos sinais clínicos que não tinham reconhecido na primeira vez. Isso demonstra a falta de conhecimento sobre a doença e os seus sinais clínicos, bem como os efeitos da pergolida, já que os proprietários notaram várias melhorias em muitos aspectos físicos de seus cavalos, em relação a fatores que não

tinham detectado no início. Acreditamos que esses resultados são de extrema importância, pois destacam uma lacuna no diagnóstico clínico e no maneio da DPIP.

Table of contents

List of figures

List of tables

List of abbreviations

ACTH- Adrenocorticotrophic hormone

ADH- Antidiuretic hormone

Alpha-MSH- α-melanocyte stimulating hormone

AVP- Vasopressin

Beta-end- β-endorphin

BID- *Bis in die* (twice daily)

CIISA- Centre for Interdisciplinary Research in Animal Health

CLIP- Corticotrophin-like intermediate peptide

e.g.- *Exempli gratia* (for example)

EDTA- Ethylenediaminetetraacetic acid

EEG- Equine Endocrinology Group

EMS- Equine metabolic syndrome

EPG- Eggs per gram

hFEC- Helminth faecal egg count

HPAA- Hypothalamus-pituitary-adrenal axis

IBM- International Business Machines Corporation

ID- Insulin dysregulation

IR- Insulin resistance

LSD- Least Significant Difference

M- Mean

MF- MiniFlotac

MM-McMaster

MR- Mean rank

n- size of sample

ODST- Overnight dexamethasone supression test

p- probability

P3- Third phalanx

PDis- *Pars distalis*

PI- *Pars intermedia*

PN- *Pars nervosa*

PO – Per os

POMC- Pro-opiomelanocortin

PPID- Pituitary *pars intermedia* dysfunction

PT- *Pars tuberalis*

PU/PD- Polyuria/polydipsia

SD- Standard deviation

SID- *Semel in die* (once daily)

SL- Suspensory ligament

SPSS- Statistical Package for Social Science

t- Ratio of the difference between the mean of the two sample sets

TRH- Thyrotropin-releasing hormone

U- Unbiased (U-statistic)

USHE- Unidade de Segurança e Honras de Estado

X^2- Chi-squared test

List of symbols and units

% Percentage

® Registered trademark

= Equals to

< Less than

> More than

≥ More or equal

± More or less

mg Miligram

µg/mL Micrograms per millilitre

mg/day Milligram per day

mg/kg Milligram per kilo

mg/500 kg/day Milligram per 500kg per day

µg/kg Mmicrograms per kilo

mg Miligram

mmHg Millimetre of mercury

ºC Degree Celsius

I. INTERNSHIP

After completing my academic journey at the Faculty of Veterinary Medicine, University of Lisbon, a curricular internship was undertaken in the areas of equine clinical practice and investigation, with the aim of completing the Master's Degree in Veterinary Medicine.

1. Experimental work

In collaboration with Professor Paula Tilley, this study was designed and then presented to the Centre for Interdisciplinary Research in Animal Health (CIISA) of the Faculty of Veterinary Medicine, University of Lisbon for further approval. After that, the recruitment process started with the aim of finding horses that would fit the inclusion criteria for this research project.

Following this, the horses included in the study where visited every month, for six months, in order to evaluate the parameters needed, which were the weight, body condition and cresty neck scores and helminth faecal egg count. On the first and last months, a more extensive evaluation of the horses was performed, which included a full physical exam, plasma ACTH measurement, measurement of blood pressure, assessment of the weight, body condition and cresty neck scores and helminth faecal egg count. Also, the owners were asked to answer a questionnaire with information regarding the horses' management, clinical signs and their evolution. For the horses in the treatment group, the plasma ACTH value was also evaluated at the end of the 2nd month, in order to assess the need for medication dosing adjustments.

All this work was conducted in ambulatory, visiting the horses in their own environment to better understand the changes during the time span of the research work.

2. Traineeship report

The internship took place at Equine Veterinary Hospital of National Republican Guard at the 4th squad of USHE (Ajuda, Lisboa) and had the duration of 5 months (from March to August).

The internship at this hospital involved accompanying Veterinary Major Daniela Teixeira in her day-to-day activities with the GNR's horses (facilities with a total of 340 animals). Daily treatments were performed, including administration of medication, wound cleaning and disinfection, monitoring clinical cases with their respective diagnoses and complementary examinations (x-ray, ultrasound, endoscopy), guidance on orthopaedic shoeing, basic dentistry procedures and medical treatment of colic (nasogastric intubations, rectal palpation, fluid therapy) (Table 2.1 and 2.2).

The hospital's clinical case casuistry is primarily dominated by musculoskeletal cases, particularly lameness. In addition to the hospital's regular activities, management of sports horses and preparation of horses for equestrian performances are also conducted.

Regarding equestrian performances, monitoring of these horses during their respective presentations was also carried out, both within the 4th squad's facilities and elsewhere. As for sport horses, I accompanied the Military week, where several horses from National Republican Guard compete in show jumping, dressage and eventing.

2.1. Table of pathologies

System/Local	Total
DIGESTIVE	
Medical colic	
Tympanic	**8**
Pelvic flexure impaction	**12**
Others	**20**
Surgical colic	**2**
Dentistry	
Regular dental work	**30**
Ulcers of the oral cavity	**7**
MUSCULOSKELETAL	
Frog infections	**12**
Hoof abscess	**20**
Cellulitis	**3**
Traumatic wounds	**9**
Chronic laminitis	**3**
Acute laminitis	**1**
Lymphangitis	**3**
Rhabdomyolysis	**5**
Fibrotic myopathy	**1**
Osteoarthritis	
Proximal interphalangeal joint	**2**
Distal interphalangeal joint	**24**
Metacarpophalangeal/Metatarsophalangeal joint	**32**
Proximal intertarsal joint	**5**
Distal intertarsal joint	**17**
Tarsometarsal joint	**10**
Digital sheath tenosynovitis	**10**
Deep digital flexor tendon contracture	**1**
Superficial digital flexor tendinitis	**3**
Deep digital flexor tendinitis	**2**
Accessory ligament of the deep digital flexor tendon desmitis	**1**
Suspensory ligament desmitis	**10**
Supraspinatus ligament desmitis	**1**
Calcaneal bursitis	**2**

RESPIRATORY	
Sinusitis	**1**
OFTALMOLOGICAL	
Chronical uveitis	**1**
Corneal ulcer	**5**
NEUROLOGIC	
Intoxication	**1**
OTHERS	
Traumatic wound (not included in the locomotor system)	**53**

2.2. Table of procedures

Procedures	Total
Intra-articular administration	
Proximal interphalangeal joint	**2**
Distal interphalangeal joint	**18**
Metacarpophalangeal/Metatarsophalangeal joint	**14**
Tarsometarsal joint	**20**
Tibiotarsal joint	**4**
Sacroiliac joint (ultrasound guided)	**4**
Back treatments	**5**
Intramuscular administration	**>80**
Intravenous administration	**>80**
Catheter placement	**5**
Fluid therapy	**5**
Nasogastric intubation	**23**
Rectal palpation	**28**
Perineural nerve block	**54**
Mesotheraphy	**1**
Laser therapy	**3**
Ultrasound	
Distal limb	**29**
Supraspinatus ligament	**1**
Abdominal	**10**
Radiography (Head, back, limb, thoracic)	**>100**
Respiratory endoscopy	**3**
Lameness exam	**>80**
Equestrian performance practices/Sport horses competitions	**73**
Orthopaedic shoeing	**20**
Bandages (Hoof/Distal limb)	**35**

II. BACKGROUND

1. Introduction

The pituitary *pars intermedia* dysfunction (PPID) is a common age-associated equine neurodegenerative disorder (Tatum et al. 2021) characterized by hyperplasic and neoplasic lesions of the pars intermedia (Miller et al. 2016) that may appear as a single adenoma or multiple smaller adenomas (McFarlane 2011). Although the pathophysiology of the disease is still not properly understood, it is thought that oxidative stress might contribute to the progressive degeneration of the inhibitory dopaminergic hypothalamic neurons (McFarlane 2014). This leads to a loss of dopaminergic inhibition of the pars intermedia lobe of the pituitary gland and overproduction of pituitary derived hormones. As a result, there is an increase on plasma concentrations of pro-opiomelanocortin (POMC) peptide and its derivatives, including α-melanocyte stimulating hormone (α-MSH), corticotropin-like intermediate lobe peptide, β-endorphin (β-END) and adrenocorticotrophic hormone (ACTH). This proliferation of hormones is associated with a variety of clinical signs and comorbidities including hypertrichosis, laminitis, epaxial muscle wastage or muscle atrophy, fat redistribution and lethargy.(Tatum et al. 2021).

PPID is also commonly known as Cushing's syndrome, despite the fact that some authors disagree with this nomination because, unlike what happens in dogs and humans, on equines this disease it's generally due to neurodegeneration and not caused by changes in the gland itself (Sojka-Kritchevsky and Johnson 2014). The term Cushing's syndrome, when properly used, refers to a real hypercortisolism, caused by hyperadrenocortisolism, something that is rarely found in the equine species (James K. Belknap and Raymond J. Geor 2016).

This disease appears slow and progressively in more than 20% of older horses (over 15 years of age) (Ireland and McGowan 2018). Its high prevalence might be associated with the fact that this disease has a chronical development and is not related to a high mortality rate (McGowan et al. 2013).

Horse's life expectancy has been increasing due to improvement in management and welfare concerns (Josie L. Traub-Dargatz et al. 2006). Therefore, it is likely that more PPID cases occur, as age is a risk factor for this disease (McFarlane 2014).

Further studies show that horses with PPID are more likely to have higher helminth fecal egg counts than healthy horses and may need more aggressive parasite prevention program (McFarlane et al. 2010).

2. Anatomy of the pituitary gland

The pituitary gland is an endocrinal structure located at the base of the brain, suspended below the hypothalamus by the infundibular stalk. This gland lies in the *sella*

turcica, separated from the brain by a fold of dura mater known as the diaphragma *sellae* (McFarlane 2011). Anatomically, it is divided in neurohypophysis and adenohypophysis. The first one is composed by the *pars nervosa* (PN) and the infundibular stem; the second includes the *pars distalis* (PDis), *pars tuberalis* (PT) and *pars intermedia* (PI) (L. C. Junqueira and José Carneiro 2017). The *pars distalis* consists of endocrine cells that synthesize, store and release hormones in response to hypothalamic releasing and inhibiting factors. These factors reach the *pars distalis* by the hypophyseal portal system that connects the capillaries of the median eminence to the capillaries of the pars distalis (McFarlane 2011). The *pars distalis* is composed of five cell types, the gonadotropes and lactotropes, that produce reproductive hormones; and the somatotropes, thyrotropes and corticotropes that release hormones regulating growth, metabolism and stress. The *pars tuberalis* is a thin band of endocrine cells that encircles the infundibular stalk and is composed of several melatonin receptors that read and decrypt daily melatonin concentrations to coordinate the output of reproductive hormones with the season of the year (McFarlane 2011). The *pars nervosa* is a collection of axons and nerve terminals that originate in the paraventricular and superoptic nuclei of the hypothalamus. The *pars nervosa* also stores and releases oxytocin and arginine vasopressin (AVP). On equines, *the pars intermedia* is composed of a single endocrine type cell, the melanotrope, that produces POMC derived peptides. The *pars intermedia* is directly innervated by the dopaminergic neurons of the periventricular nucleus of the hypothalamus.

3. Physiology of the pituitary gland

The activity of the hypothalamus and pituitary is seasonal (related to the photoperiod), with greater hormone production, in particular adrenocorticotropic hormone and alpha melanocyte stimulating hormone, occurring in late summer and early autumn, when the amount of hours of daylight begins to decrease (McFarlane et al. 2004; Beech et al. 2009; Schreiber et al. 2012).

The PDis is formed by several types of endocrine cells, including the corticotrope, whose function is to synthesize, store and release hormones according to the control exerted by the hypothalamus, being under negative feedback from glucocorticoids. In the PDis, corticotropes produce ACTH. The PT is closely linked to the seasonality of the release of reproductive hormones according to the input of its numerous melatonin receptors. The PN serves as a reservoir of oxytocin and antidiuretic hormone. The PI is formed by a single type of cell, the melanotrope, and is controlled by dopaminergic neurons originating in the hypothalamus and ending in the PI itself.

Melanotropes produce POMC if the thyrotropin-releasing hormone (TRH) acts on D2 dopaminergic receptors. However, this production is inhibited if dopamine acts on the receptors (McFarlane 2011; L. C. Junqueira and José Carneiro 2017). These cells are also responsible for the production of POMC-derived peptides through their enzymatic cleavage

by pro-convertases 1 and 2. Pro-convertase 1 is produced by melanotropes and corticotropes and cleaves POMC into ACTH, while pro-convertase 2 is produced only by melanotropes and its function is to cleave ACTH into α-MSH and corticotropin-like intermediate peptide (Carmalt et al. 2018).

In the PDis and the PI, β-endorphins and gamma-lipotropins are produced through the cleavage of beta-lipotropin (Brian K. Petroff and Deborah S. Greco 2021).

In summary, cleavage of POMC in the PDis results in most ACTH production in a healthy horse (McFarlane 2011). It also controls the production of glucocorticoids by the *fasciculata* and *reticularis* zones of the adrenal gland (Brian K. Petroff and Deborah S. Greco 2021). Furthermore, the cleavage of POMC results in the production of β-endorphins, endogenous opioid agonists with analgesic, pain-reducing and inflammation-reducing properties and of α-MSH (McFarlane 2014). The latest is the primary product of the PI and has potent effects on stress, inflammation (Oktar et al. 2004; Catania et al. 2010), metabolism itself, and obesity (regulates glucose and lipid use, in combination with leptin and insulin, inducing anorexia by promoting a satiety sensation which has effect on the hypothalamus) (Zhang and Scarpace 2006; Denis et al. 2014; Sobrino Crespo et al. 2014).

The physiology of the pituitary gland is summarized in Figure 1.

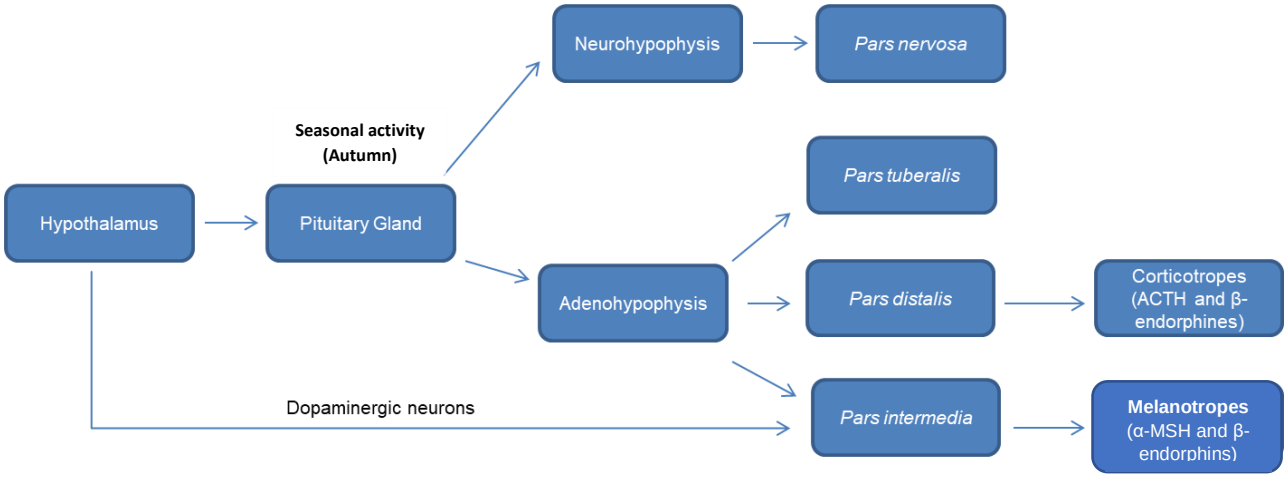


Figure 1 – Simplified diagram of the anatomy and physiology of the pituitary gland. Adapted from McFarlane, D. (2011). Equine pituitary pars intermedia dysfunction (McFarlane 2011)

4. Pituitary *pars intermedia* dysfunction

PPID is a progressive lifelong neurodegenerative disease, common in older equids. A loss of dopaminergic inhibitory output to the pituitary pars intermedia results in increased plasma concentrations of multiple POMC derived peptides, including ACTH, α-MSH, β-endorphin and corticotrophin-like intermediate peptide (CLIP) (Ireland and McGowan 2018; Tatum et al. 2021).

This disease is commonly associated with specific clinical signs like hypertrichosis and abnormal hair shedding, laminitis, epaxial muscle wastage or muscle atrophy, weight

loss and fat redistribution, polyuria/polydipsia (PU/PD), lethargy or depression and recurrent infections (including parasitic infections that might result in higher helminth fecal egg counts) (Ireland and McGowan 2018; Burns et al. 2018; Tatum et al. 2021). The most prevalent are hypertrichosis, laminitis, epaxial muscle wastage (“sway back”) and weight loss, the latter in older horses.

The syndrome was originally referred to as equine Cushing disease due to similarities to Cushing disease in humans and dogs, in which there is excessive and autonomous secretion of POMC–derived peptides, including ACTH, resulting in pituitary-dependent hyperadrenocorticism. However, in equids, pituitary adenomas arise from the *pars intermedia* (intermediate lobe) of the pituitary gland, unlike in humans and dogs, in which pituitary adenomas commonly develop from the anterior lobe (pars distalis, adenohypophysis). As a result, in the horse, hormones from the pars intermedia are excessively secreted as described ealier. PPID therefore seems to be a more appropriate term to describe the syndrome because, unlike humans, adrenocortical hyperplasia and hypercortisolemia are not consistent findings in affected horses.

4.1. Clinical signs

The progression of clinical signs in PPID is slow and insidious, which makes it very difficult to ensure an early diagnosis (Orth and Nicholson 1982; Millington et al. 1988; Donaldson et al. 2002: 20052005; McFarlane et al. 2005; Perkins et al. 2010), making it necessary to resort to complementary diagnostic tests, such as endocrine tests.

Clinical signs of PPID in aged horses and ponies include hypertrichosis, poor haircoat, laminitis, PU/PD, muscle wasting, weight loss/repartitioning, docility, lethargy, hyperhidrosis, narcolepsy (and other signs of CNS dysfunction, including seizure), blindness, decreased responsiveness to painful stimuli, increased appetite, and recurrent infections. Although there are many clinical signs associated with PPID, only a subset of the signs are typically manifested in an affected individual, perhaps due to differences in the secretion profile of POMC-derived peptides or to the degree of compression of adjacent neuroendocrine tissues (Burns et al. 2018).

It is also known that the number of presented clinical signs is related to the increase in ACTH and α-MSH plasmatic levels (McGowan et al. 2013).

4.1.1. Hypertricosis

Hypertrichosis (55%–80%) is considered the most frequent clinical sign in PPID horses, although these estimates may be biased due to the increased tendency to test aged horses displaying haircoat abnormalities for PPID (Burns et al. 2018).

Although initially ‘’hirsutism’’ was the term used to describe long and curly hair or abnormal hair shedding, nowadays the appropriate term is in fact ‘’hypertricosis’’, as the

PPID typical phenotype is due to the capillary persistence in the anagen phase, unlike the excess of androgens that occurs in human Cushing's syndrome (Innerå et al. 2013).

McFarlane 2014 proposes that hypertricosis might be caused by an excess of prolactin production, since prolactin is also under dopaminergic control and, apart from intervening in the reproductive system (Thompson and Oberhaus 2015), it is involved in hair shedding (lower concentrations of prolactin in the winter, and higher values in the summer, when hair falls). Prolactin can be increased initially in PPID, due to dopamine deficiency, and later this hormone's production may cease thanks to the *pars distalis* compression (including lactrotropes), which would explain why haircoat shedding doesn't occur in more advanced stages of the disease.

Hypertrichotic horses have a long, thick, often curly haircoat and fail to shed in a complete or timely manner (Schott 2002; Frank et al. 2006; Burns et al. 2018) especially in more advanced phases of the disease. Other haircoat abnormalities may include retention of long hairs in the jugular furrow, in the distal limbs, within the intermandibular space, or along the ventral abdomen, which are mostly observed in early phases of PPID (Schott 2002; Frank et al. 2006).

McGowan et al. 2013 conducted a study that concludes that the presence of hypertrichosis, when associated with other specific clinical signs of PPID (reported in the clinical history or the clinical exam) was considered a gold standard for PPID diagnosis.

4.1.2. Hyperhidrosis

Excessive sweating (hyperhidrosis) and, less commonly, lack of sweating (anhidrosis) are reported in horses with PPID, even in clipped horses (McFarlane 2011; Burns et al. 2018). Meanwhile the mechanisms underlying abnormal sweating remain unclear (McFarlane 2014).

4.1.3. Polyuria/polydipsia

Polyuria and polydipsia have been reported in up to 76% of horses diagnosed with PPID. This condition is more easily detected in horses housed in stalls than at pasture, questioning the real accuracy of PU/PD in horses with PPID (Burns et al. 2018). The pathogenesis behind the development of PU/PD is still uncertain. It has been speculated that compression of the *pars nervosa* by an expanding *pars intermedia* adenoma may result in decreased secretion of vasopressin (AVP, antidiuretic hormone [ADH]) (Loeb et al. 1966; Heinrichs et al. 1990). As some horses with PPID are hyperglycemic, this can induce osmotic diuresis. Also, glucocorticoids can increase the glomerular filtration rate and may contribute to diuresis, but hypercortisolemia is also inconsistent in horses with PPID. It is important to rule out other differentials for PU/PD, including neurogenic diabetes insipidus, nephrogenic diabetes insipidus, psychogenic polydipsia, and hyperglycemia of various origins (chronic

pain, pancreatic disease, pheochromocytoma, insulin resistance) (Schott 2002; Messer and Johnson 2007; McFarlane 2011; Burns et al. 2018).

4.1.4. Suspensory ligament (SL) degeneration

In older horses with pituitary *pars intermedia* dysfunction, suspensory ligament (SL) degeneration is common. Suspensory ligament degeneration leads to fetlock hyperextension and is common in several breeds of horses (Hofberger et al. 2015). Horses with pituitary pars intermedia dysfunction also frequently present fetlock hyperextension. Although cortisol concentrations are not consistently elevated in horses with PPID, degeneration of the SL may also be associated with long term exposure to intermittently high cortisol levels (Hofberger et al. 2015; Hofberger et al. 2018).

4.1.5. Endocrine Laminitis

Laminitis and metabolic abnormalities including hyperglycemia and hyperinsulinemia also occur in approximately 30% of horses with PPID. It is now thought that endocrinopathic laminitis occurs in horses with PPID due to concurrent insulin dysregulation or equine metabolic syndrome (EMS), rather than as a direct result of PPID itself (Hart et al. 2020).

Endocrine laminitis has a chronic and subclinical character. It is identified in association with PPID or EMS and constitutes about 70% to 90% of all cases of laminitis encountered in clinical practice (Donaldson et al. 2004; Karikoski et al. 2011; Karikoski et al. 2016). Of these cases, between 24% (Tim D. G. Watson 1998; McGowan and Neiger 2010) and 80% are reported in clinical case series and 13% in epidemiological studies (McGowan et al. 2013). There is an abnormal growth of the hoof due to the separation of the third phalanx (P3) from the sensitive lamina with its sinking and rotation favored, respectively, by the vertical force exerted by the animal's body weight in P3 and by the force exerted by the deep digital flexor tendon in a caudodorsal direction.

Laminitis in the context of PPID is not usually caused by hypercortisolaemia (Asplin et al. 2007; Nourian et al. 2009; Frank and Tadros 2014), as only a minority of horses (about 20%) (Schott 2002) have adrenal gland hypertrophy, this being due to hyperinsulinemia. It is now known that supraphysiological insulin concentrations are an important risk factor for the development of laminitis and are indicative of a poor prognosis (Asplin et al. 2007; McGowan et al. 2010; De Laat et al. 2012; James K. Belknap and Raymond J. Geor 2016). The age of the horse itself constitutes a risk factor for the development of laminitis (Polzer and Slater 1997; Karikoski et al. 2011). Although its origin is not yet fully understood, there are several mechanisms through which an attempt is made to explain the development of laminitis by the presence of supraphysiological concentrations of insulin, either from insulin dysregulation (ID) or insulin resistance (IR) (Burns et al. 2018). It should be noted that laminitis, being

subclinical, may be present for months, or even years, until it is clinically identifiable (Schott 2002; Donaldson et al. 2004; McFarlane 2011; Durham et al. 2014).

The most frequent clinical manifestation of this type of laminitis involves abnormal hoof growth, presence of prominent growth rings with palmar/plantar divergence and convergence in the dorsal area, enlargement of the white line (particularly in the toe area) with possible development of white line infection, convexity of the sole, contracture of the heels and “Aladdin's slipper” appearance, in which the dorsal wall of the hoof becomes concave and the toe begins to rotate in the dorsal direction. These horses are generally painless, but pain can be demonstrated by shifting weight from the toe to the heels (most often on the forelimbs), reluctance to walk, and intermittent digital pulse after exercise on hard ground (caused by bruising). These episodes of pain tend to be less intense than those of acute laminitis and can be triggered by ingesting forage with high sugar content or during the autumn season, when there is a greater production of POMC derivatives (James K. Belknap and Raymond J. Geor 2016).

It is possible that horses with PIPD, or even EMS, are predisposed to develop laminitis from causes other than endocrine, due to the fact that their foot structure is chronically altered, mainly in the two cranial thirds. The change in these structures often result in the occurence of white line disease and, consequently, subsolar abscesses (James K. Belknap and Raymond J. Geor 2016).

4.1.6. Muscular atrophy/ Weight loss or fat redistribution

Studies show that body condition is positively related to the α-MSH plasmatic concentration (Donaldson et al. 2004; Beech et al. 2009). Also, fat excess is associated to insulin resistance causing chronical inflammation and inhibiting the normal mitochondrial function, leading to oxidative stress, one of the main causes of PPID (McFarlane 2011).

So, it is not clear yet if abnormal fat distribution is a consequence of PPID or a predictor factor for this disease (McFarlane 2011).

Abnormal fat redistribution is observed in 15 to 30% of PPID horses (Schott II et al. 2001), mainly in the supraorbital, neck, base of the tail and inguinal regions (Carter et al. 2009).

Weight loss or repartitioning is reported in the majority of horses with PPID (up to 88%) and is often an early clinical sign; however, some horses may be obese or in good body condition. Affected horses often have a weak and pendulous abdomen, a sway back, and a poorly developed topline (Kirkwood et al. 2022; Gris et al. 2023). The potbellied appearance is probably the result of lack of tone in the abdominal musculature. The muscle wasting along the dorsal midline and elsewhere results in prominence of the *tuber coxae* and *tuber sacrale* regions. It is unclear what the effect of different POMC-derived peptides is on equine energy metabolism or muscle atrophy. Weight loss in some horses may be the result

of other conditions associated with aging (such as dental problems, poor nutrition, or chronic disease) or PPID (opportunist infections including parasitism secondary to immunosuppression) rather than the direct effect of an abnormally regulated hypothalamus-pituitary-adrenal axis (HPAA) on body condition (Hart et al. 2020). Glucocorticoids, in addition to being immunomodulatory, can have catabolic effects on skeletal muscle; however, the role of increased concentration of cortisol on weight loss in PPID is unclear. Muscle tissue from horses with PPID shows selective atrophy of type 2 muscle fibers and differential expression of m-calpain (a major proteolytic system that may play a role in the development of muscle atrophy in horses with PPID); however, the specific role of POMC hormones on muscle atrophy has not been investigated (Banse et al. 2021; Kirkwood et al. 2022).

The expanded supraorbital adipose tissue often observed in horses with PPID results from fat redistribution (Morgan et al. 2018). This finding may be observed in horses that do not exhibit generalized obesity. Likewise, some horses at higher body condition scores may have prominent nuchal ligament ("cresty neck") and tailhead adipose tissue accumulation (regional adiposity) (Nicholas Frank et al. 2022).

4.1.7. Letargy

Increased docility and letargy in affected horses is often reported by owners and clinicians. This can be due to several causes such as insulin resistance, a concomitant disease or high concentrations of β-endorphin (Millington et al. 1988; McFarlane 2011). An increased plasma and cerebrospinal fluid concentration of β-END is observed in horses with PPID, which may explain the docility and lethargy in some of these animals. The increase of this neuropeptide can also justify the decreased responsiveness to painful stimuli and corneal stimulation that is observed in affected animals. Corneal ulcers may develop both with age and with PPID, which may explain the frequency of chronic ulcerative keratitis in these horses (Miller et al. 2013).

4.1.8. Imunossupression

4.1.8.1. Recurrent infections

Immunosuppression is a consequence of PPID that is manifested by the occurrence of opportunistic secondary infections, such as sinusitis, pneumonia, dental abscesses, skin and ocular infections (Schott 2002; McFarlane 2011).

It was thought that changes in immune response could be due to hypercortisolism, but this isn't a consistent condition in these horses, because the ACTH that is excessively produced in PPID is considered inert (Orth and Nicholson 1982; J. Beech et al. 2011).

The α-MSH is a hormone with severe immunomodulatory effect that in other species was proven to decrease the production of superoxides and the migration and adhesion of

neutrophils, therefore reducing these cells' functions in general (McFarlane et al. 2015). Insulin concentration also influences neutrophils' function and the increase of its concentration is directly proportional to the production of superoxides (Yano et al. 2012). McFarlane et al. 2015 showed that secondary infections were due to changes in neutrophils' performance. Also, changes in the production of superoxide occurred, when in the presence of either insulin or α-MSH, even though there was no significant correlation. Yet, the ratio between pro-inflammatory factors (insulin) and anti-inflammatory factors (α-MSH) showed a strong correlation with the production of superoxides. However, further studies are needed to understand the specific relation between these hormones and neutrophil dysfunction.

4.1.8.2. Parasitic infections

McFarlane et al. 2010 studied the increased susceptibility of horses with PPID to parasitism. The results didn't show any specific cause. Although the increase of POMC–derived peptides had an immunosuppressor effect, it was related to an increase in Th2 cytokines (related to parasite resistance in other species) rather than Th1 (related to susceptibility).

Nonetheless, they also concluded that, regardless of the origin of this predisposition, horses with PPID have higher helminth fecal egg counts and shorter time to egg reappearance when compared to healthy horses, which puts in danger the health of other animals around them.

4.1.9. Reduced fertility

Abnormal estrus cycles and infertility have been reported in horses with PPID. This may be a direct or indirect effect of PPID, caused by uterine infections related to immunosuppression, or due to a deficient output of reproductive hormones like gonadotropin-releasing hormone and prolactin, thanks to a decrease in dopaminergic regulation (McFarlane 2011).

4.1.10. Neurological signs

Although further studies are necessary regarding this topic, neurologic impairment has been reported in horses with PPID (6 to 50% of the cases) and includes blindness, seizures, and narcolepsy (McFarlane 2011).

Bilateral blindness may arise due to optic chiasm compression by the enlargement of the pituitary gland (Kolk 1997). In that case, it will have a later appearance, since the adenoma's growth is normally slow and progressive. However, in 2001, Takeuchi (Takeuchi 2001) reported a case of bilateral blindness in a young horse with PPID and suggested, by the precocity of the clinical signs, that the tumor was not formed by melanotropes, but rather by glil-like cells or ACTH-like cells, which are considered true corticotropic cells. The size

reduction of the *pars intermedia* would benefit the nervous structures compressed by it, yet there aren't any known cases where PPID medication had this effect (Madrigal et al. 2018).

Another neurological sign in horses with PPID is narcolepsy or sleep disorders. Narcolepsy may occur as a result of reduced orexin activity in the hypothalamus. Orexins (hypocretins) are peptide neurotransmitters expressed in the lateral hypothalamus (orexinergic neurons) that are important in the sleep-wake cycle; the absence of orexin function results in narcolepsy. Horses with PPID have been shown to have decreased cerebrospinal fluid concentrations of hypocretin (orexin) (Chemelli et al. 1999; McFarlane 2007).

4.1.11. Laboratory findings

The results shown by McGowan et al. (2013) do not show the existence of haematological or biochemical changes which can be specifically attributed to PPID and the authors concluded that the changes could be due to inflammation or any other concomitant disease rather than a specific sign of PPID.

The haematological variations more frequently described are slight neutrophilia and lymphopenia. Hyperglycemia occurs in 58 to 94% of the horses with PPID (Keen et al. 2010), being considered the most common biochemical change. Several studies also report hyperglycemia as a laboratory finding in PPID as well as hyperinsulinemia, hypertriglyceridemia and high faecal egg count (Love 1993; McFarlane 2011; Durham et al. 2014; Hart et al. 2021).

4.2. Diagnosis

The diagnosis of PPID in many cases can be suggested by history, clinical signs, and preliminary laboratory results.

PPID is very often underdiagnosed, as owners attribute many of its clinical manifestations to old age alone. There are a number of different endocrine laboratory tests than can be used to diagnose this disease. However, the presence of three or more clinical signs, associated with the measurement of plasma concentrations of ACTH reporting to the seasonal reference range, establishes the best way to proper diagnose PPID (McGowan et al. 2013; McGowan et al. 2013).

When the clinical signs are used as a diagnostic tool, hypertrichosis has an important role, as it is considered almost as pathognomonic of PPID and highly predictive of the existence of *pars intermedia* adenomas (Schott 2002; Frank et al. 2006).

The evaluation of the clinical signs is always useful but can sometimes be nonspecific (Miller et al. 2008) and it is necessary to carry out laboratorial tests not only to assist in the diagnosis of equivocal cases, but also to identify possible coexisting diseases (such as Equine metabolic syndrome) (Durham et al. 2014). The ambiguous cases of PPID belong to

the called grey zone, which is characterized by cases of horses that present clinical signs suggestive of PPID but hormonal values within the normal range or animals without clinical signs but hormonal values above the normal range (Hart et al. 2021). The grey zone is then a transition period between the healthy state and declared disease, as POMC–derived peptides are continually produced over time (McGowan et al. 2013).

The choice of laboratorial test has been adapted through time as new studies and conclusions are made on this matter.

4.2.1. Baseline ACTH and α-MSH concentration

The measurement of ACTH and α-MSH plasma concentrations has shown to be helpful in PPID diagnosis.

ACTH is physiologically produced by the corticotropes of the *pars distalis,* whereas α-MSH is primarily a product of the *pars intermedia,* so an abnormal increase in its concentration is highly suggestive of PPID*.* When in the presence of PPID, ACTH is also released from the *pars intermedia* (McFarlane 2011). The physiological increase of α-MSH (also associated with the photoperiod) is thought to be a natural mechanism of preparation for winter, when, naturally, there is a lower availability of food, leading to an increase in fat storage, and this mechanism is exacerbated in PPID (McFarlane et al. 2004).

Copas and Durham (2012) suggest autumn season (from august to october) as the best time to analyse ACTH, as at this time the plasmatic concentration physiologically increases more in horses with PPID than in healthy horses. Furthermore, they determined cut-off values for PPID diagnosis, suitable for each season. For non-autumn months (november to july) the ACTH plasmatic concentration in horses with PPID is >29 µg/mL and in the autumn months >47 µg/ml. McGowan, Pinchbeck and McGowan (2013) (McGowan et al. 2013), in addition to supporting the use of these benchmarks, enhance their added value to the diagnose of PPID during autumn, since this season naturally stimulates the hypothalamic–pituitary–adrenal axis in the same way that exogenous TRH stimulates this axis (McFarlane et al. 2006; Beech et al. 2007).

It's also important to have in mind that the screening of endocrinal markers, such as ACTH, glucose or insulin is affected by alpha-2 agonist sedatives (Alexander and Irvine 2001). Besides, ACTH has been reported to be affected by stress and pain, exercise and ingestion of feed (Tatum et al. 2020).

The plasma concentration of ACTH varies with the photoperiod, and its increase is associated with a decrease in the number of hours of light per day after the summer solstice (Beech et al. 2009). This sazonal biorhythm is mediated by melatonin, a hormone produced during the night by the pineal gland that acts on receptors of the *pars tuberalis*, controlled by the production of POMC, whose peak and amplitude varies with geographic location (McFarlane 2011).

In contrast to ACTH, α-MSH has the advantage of not being affected by stressful situations, concomitant diseases or intense physical exercise (McFarlane et al. 2004), which facilitates its interpretation, but in refrigerated blood it is only stable for 8 hours and it needs treatment (centrifugation, plasma separation and eventually freezing) before it can be transported, whereas ACTH is stable for 48 hours in refrigerated blood (McFarlane et al. 2004; Rendle et al. 2015).

In several studies, α-MSH was shown to be a better diagnostic choice than ACTH (McFarlane 2011; Spelta 2015). A greater sensitivity and specificity was obtained in an epidemiological study carried out in the fall (McGowan et al. 2013), as the results showed fewer false positive results in normal horses and fewer false negatives in horses with PPID (Beech et al. 2009). However, there is no commercial test that allows an easy evaluation of this hormone, therefore ACTH remains the most used diagnostic parameter.

4.2.2. TRH stimulation test

Initially, this test was developed to measure serum cortisol concentration ten and thirty minutes after TRH administration. It has a sensitivity of 92% and its specificity and positive predictive values (probability of an evaluated individual with a positive result being really sick) and negative predictive values (probability of an evaluated individual with a negative result being really healthy) are increased when it is associated with the dexamethasone suppression test (Frank et al. 2006), but it involves multiple blood draws over 24 hours (McFarlane 2011).

According to Beech and Garcia (1985), the administration of TRH would lead to an increase in serum cortisol concentration by 30% to 50%, but recent studies state that the increase in cortisol in horses with PPID rarely occurs when ACTH is increased (J. Beech et al. 2011; McFarlane 2011) due to the fact that ACTH produced in excess is biologically inert.

Currently, ACTH is the parameter to measure after TRH administration. TRH is administered intravenously at a dose of 0,5 mg (for horses weighing less than 250 kg) or 1 mg (for horses weighing more than 250 kg) (Hart et al. 2021) and leads to increased plasma concentration of ACTH and α-MSH in healthy horses and to a greater extent in those with PPID. The plasma concentration of ACTH peaks between 2 and 10 minutes after the administration of TRH and then gradually declines over an hour until it returns to baseline (McFarlane et al. 2006; Beech et al. 2007; J. Beech et al. 2011; Jill Beech et al. 2011; Funk et al. 2011). A cut-off of > 36 µg/ml is associated with a sensitivity of 94% and specificity of 78% in the thirty-minute measurement (J. Beech et al. 2011). It is not recommended to perform this test until 12 hours after eating cereal grains or until 24 hours after performing an oral glucose test and its administration may have transient side effects including coughing, Flehmen response and yawning (Hart et al. 2021).

Performing this test is recommended by the Equine Endocrinology Group (EEG) (Hart et al. 2021) cases where the basal plasma ACTH concentration is inconclusive, but since TRH production is also influenced by seasonality, it is not recommended that this test be performed in the autumn. It is necessary to create adapted reference intervals that allow the interpretation of results throughout the year (Spelta 2015).

Although so far this is the test with the best diagnostic sensitivity, it is generally only used in university or referral hospitals due to the fact that TRH is an expensive substance and is difficult to obtain.

4.2.3. Overnight dexamethasone suppression test (ODST)

The overnight dexamethasone suppression test (ODST), is based on the negative feedback that the excess of glucocorticoids has on pituitary-adrenal axis, resulting in the inhibition of ACTH production by adenohypophysis (McFarlane, 2011). It is not a frequently performed test because the melanotropes of the PI do not have glucocorticoid receptors, so they're not under the control of the pituitary-adrenal axis (Miller et al. 2008). Since most of the ACTH in PPID arises from PI production, its plasma concentration may remain unchanged at the end of the ODST (Dybdal et al. 1994). The amount of ACTH produced in excess is biologically inert, with a dissociation between this hormone and the concentration of cortisol (McFarlane 2011), which makes this test inappropriate for PPID diagnosis. Also, the sensitivity of the pituitary-adrenal axis to the action of corticosteroids decreases with advancing age (the older the horse, the greater the cortisol concentration in the beginning and the end of ODST) (Donaldson et al. 2005).

Furthermore, performing this test in horses with suspected PPID is always a risk since one of the clinical signs is laminitis, which can be exacerbated by the administration of dexamethasone (Rendle 2013).

Nevertheless, if this test is performed, it is considered that an elevated cortisol concentration, 18 to 20 hours after dexamethasone administration is indicative of PPID (Durham et al. 2014).

4.2.4. Other tests

Some other tests like oral domperidone challenge test or combined dexamethasone suppression/TRH stimulation test with cortisol measured used to be performed to diagnose PPID. However, recent guidelines for PPID treatment (Hart et al. 2021) consider those test no longer recommended. Also, hormonal tests such as ACTH stimulation test, baseline cortisol concentration and diurnal cortisol rhythm are not appropriate to diagnose this disease.

4.3. Pharmacological treatment

When the diagnosis of PPID has been made, a pharmacological treatment plan can be instituted alongside with diet changes and a management plan for these horses. There are some drugs available for the treatment of this disease, but, according to the EEG (Hart et al. 2021), the most efficient is pergolide mesylate.

The protocol described in Figure 2 should be followed, being evaluated on a case-by-case basis in order to decide how to continue the treatment.

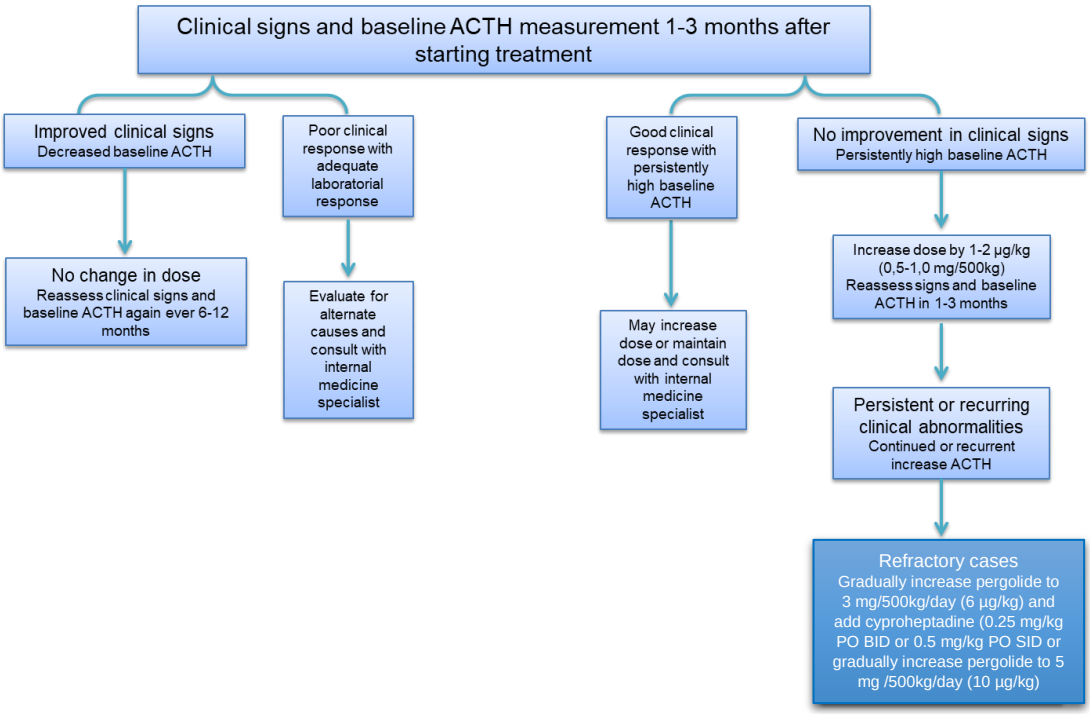


Figure 2- Protocol for treatment and monitoring horses with PPID. Adapted from Hart et al., 2021. Recommendations for the Diagnosis and Treatment of Pituitary Pars Intermedia Dysfunction (PPID) (Hart et al. 2021)

4.3.1. Pergolide mesylate

Pergolide mesylate, an ergot-derived dopamine receptor agonist which leads to downregulation of the production of POMC-derived peptides, is the treatment of choice. Statistically significant reduction in plasma ACTH is achieved 24h after commencement of treatment. The recommended starting dose is 0,002 mg/kg per day (1mg/day for a 500 kg horse). After 2 months, if there is inadequate improvement in the laboratory tests or no evidence of clinical improvement, the dose can be increased to 0,004mg/kg per day (2 mg/day for a 500 kg horse). Some authors recommend an initial dosage of 0,001 mg/kg with incremental increases of 0,001 mg/kg every 3-8 weeks until clinical resolution is achieved and in order to reduce the occurrence of side effects. An initial side effect of this treatment may be inappetence, where the dose can be halved for 1-2 weeks and then gradually increased. Depression and diarrhea may also occur, in which case the drug should be discontinued for 2-3 days and then recommenced at half dose, following which 0,5 mg

increments can be carried out every 2-4 weeks. Cardiac side effects have not been recognized in horses (Spelta and Axon 2012; Secombe et al. 2018; Tatum et al. 2020).

4.3.2. Cyproheptadine

Cyproheptadine is a seratonin antagonist that, due to its poor results, is rarely used in monotherapy (McFarlane 2011; Rohrbach et al. 2012). The EEG (Hart et al. 2021) suggests the combination of this drug with pergolide mesylate in refractory cases: gradually increasing pergolide to 3 mg/500 kg/day (6 µg/kg) and adding cyproheptadine (0,25mg/kg PO BID or 0,5 mg/kg PO SID).

4.3.3. Cabergoline

It is a dopamine agonist and synthetic derivative of the ergot alkaloid with long-lasting action (Godbout et al. 2010; Godoy and de la Fuente 2022). Godoy and de la Fuente (2022) effectively showed the effect of this medication on a mare with PPID with total improvement of the clinical signs of the disease and analytical parameters**.**

4.3.4. Medications no longer in use

4.3.4.1. Bromocriptine

It is a synthetic ergot alkaloid and dopamine receptor agonist, acting on D2-type receptors (Arana-Valencia et al. 2018; Fortin et al. 2020). Although it has been studied in horses it is rarely used nowadays (Arana-Valencia et al. 2018).

4.3.4.2. Trilostane

It is an inhibitor of the synthesis of corticosteroids by the adrenal glands, which is not useful in most cases of PIPD, since the occurrence of adrenal gland hyperplasia and hypercortisolemia by this route is rare (McFarlane et al. 2006; J. Beech et al. 2011), and because it has no effect on excessive pituitary hormone production.

5. Helminth faecal egg count

Parasite burden in horses with PPID can vary and may be influenced by several factors, including the horse's age, management, environment, and overall health. Since PPID affects the pituitary gland in older horses, it can lead to hormonal imbalances, immune system changes, and alterations in metabolism. These changes may influence the horse's susceptibility to gastrointestinal parasitism (McFarlane et al. 2010).

Studies have shown mixed results regarding the relationship between PPID and parasite burden in horses (Kirkwood et al. 2022). Some research suggests that horses with PPID may have a higher parasite burden compared to healthy horses (McFarlane et al.

2010), while other studies have found no significant difference in parasite burdens between the two groups (Christen et al. 2018).

Equine helminth fecal egg count (hFEC) is a diagnostic test used in veterinary medicine to assess the level of internal parasite burden in horses. Helminths are parasitic worms that can infect the gastrointestinal tract of horses. The hFEC is an important tool to monitor and manage parasite infections. Regular monitoring of hFEC is essential for implementing effective parasite control programs that help maintain the health and well-being of horses (Lester and Matthews 2014).

5.1. Helminth faecal egg count techniques

The McMaster technique is a widely used method for quantifying the number of parasite eggs in a faecal sample. It is commonly employed to estimate the faecal egg count (FEC) of gastrointestinal parasites, such as strongyles, in horses (Noel et al. 2017). This technique allows veterinarians and horse owners to assess the level of parasite burden in the horse's gastrointestinal tract and determine appropriate deworming strategies. It involves using a specialized device called a McMaster counting chamber, which has two chambers with grids (Dias de Castro et al. 2017). Using this technique, the number of eggs counted is multiplied by 50 (detection limit), which takes into account the dilution and volume of faeces examined. This calculation gives the hFEC, expressed as eggs per gram of faeces (EPG) (Franco et al. 2015). This technique is widely used due to its relative simplicity and accuracy in quantifying parasite eggs in faecal samples. It allows for more targeted and strategic deworming protocols, reducing the risk of anthelmintic resistance and promoting better parasite control in horses (Went et al. 2018).

The Mini-FLOTAC technique involves using a specific device called the Mini-FLOTAC apparatus, which consists of a faecal sample container and a counting chamber (Barda et al. 2013). Similar to other hFEC techniques, the number of eggs counted is multiplied by 25 to calculate the hFEC, expressed as eggs per gram of faeces (EPG).This technique has gained popularity due to its improved accuracy and efficiency in detecting parasite eggs, including those of small or low egg-shedding parasites. As with any diagnostic test, it's crucial to follow proper sample collection and processing protocols to obtain reliable and meaningful results (Cringoli et al. 2017).

While both techniques aim to achieve similar goals, they have some differences in terms of their design and performance. The McMaster technique is a standard and well-established method, but it may have limitations in detecting low levels of parasite eggs or eggs of small-sized parasites (Went et al. 2018). While, the Mini-FLOTAC technique is an advancement of the McMaster technique and is known for its increased sensitivity and accuracy, particularly in detecting low-level infections and smaller parasite eggs (Cringoli et al. 2017). Both techniques have their advantages and limitations, and the choice of method

may depend on the specific needs of the veterinary practice or research project. The Mini-FLOTAC technique is considered an improvement over the McMaster technique, particularly in situations where detecting low-level infections or small parasite eggs is crucial for effective parasite control. However, the availability of equipment, resources, and the level of expertise in performing the technique also play a role in the selection process.

III. EXPERIMENTAL WORK:

CONTRIBUTION TO THE EVALUATION OF THE BENEFITS OF PERGOLIDE IN THE TREATMENT OF EQUINE PITUITARY PARS INTERMEDIA DYSFUNCTION (PPID): REDUCTION OF HELMINTH FAECAL EGG COUNTS (HFEC) AND INCREASED OWNER AWARENESS

1. Introduction

Due to the increment in life expectancy and consequent aging of the equine population, geriatric equine medicine is becoming increasingly important. Pituitary pars intermedia dysfunction is the most common endocrine disorder of older equids (especially ≥ 15 years of age), which makes it considerably relevant in equine practice (Ireland and McGowan 2018; Fortin et al. 2021; Kirkwood et al. 2022).

PPID is a progressive lifelong neurodegenerative disease (van Proosdij and Frietman 2022). A loss of dopaminergic inhibitory output to the pituitary pars intermedia results in increased plasma concentrations of multiple POMC derived peptides, including adrenocorticotrophic hormone, α-melanocyte stimulating hormone, β-endorphin and corticotrophin-like intermediate peptide (Ireland and McGowan 2018; Horn et al. 2019; Tatum et al. 2021).

There are several different endocrine laboratory tests which can help to diagnose this disease (Tatum et al. 2021). The basal tests measure either plasma ACTH or α-MSH concentrations and the dynamic tests include overnight dexamethasone suppression test and thyrotropin-releasing hormone stimulation test. The ODST has been found to have reduced sensitivity, particularly in cases of early or mild disease, particularly during autumn. The clinicopathological test of choice for the diagnosis of PPID is the basal endogenous ACTH concentration (Secombe et al. 2018; Durham et al. 2020). The available evidence demonstrates that the measurement of basal ACTH is highly specific and has good sensitivity for the diagnosis of PPID in horses and ponies with clinical signs consistent with the disease (Tatum et al. 2021). In one study, the greatest difference in plasma ACTH between PPID-affected and non-PPID-affected horses occurred between August and October (Copas and Durham 2012). Therefore, plasma basal ACTH is frequently used for PPID diagnosis and in another study the upper reference range limit was established as 77.4 µg/ml in autumn and 29.7 µg/ml for the rest of the year (McGowan et al. 2013; McGowan et al. 2013). Meanwhile, ACTH has been reported to be affected by stress and pain, exercise and ingestion of food (Tatum et al. 2020).

The gold standard treatment for PPID is pergolide mesylate, an ergot-derived dopamine receptor agonist which leads to downregulation of the production of POMC-derived peptides (Jacobson et al. 2014; Meyer et al. 2022). Statistically significant reduction in plasma ACTH is achieved 24h after the beginning of treatment (Rendle et al. 2019). Average life expectancy following PPID diagnosis has been reported to be 9.8 years, so pergolide is likely to be taken for a considerable number of years. There is some evidence that it may have a protective effect against further neurodegeneration caused by oxidative stress, improving the prognosis of PPID cases (Tatum et al. 2020).

In a systematic review, improvement in at least one clinical sign following the beginning of pergolide treatment ranged from 40% to 100% with a high proportion of cases (>76%) showing clinical improvement in the majority of studies. Endocrinological improvement can be defined as a post-treatment reduction of > 50% in basal ACTH (Tatum et al. 2020). However, a recent study has reported treatment failure with more than half of the animals appearing to not be receiving the recommended dosage of pergolide. Therefore, it is prudent to investigate the owner's medication compliance and how to advise on overcoming any barriers (Hague et al. 2021).

The clinical signs of PPID include hypertrichosis and abnormal hair shedding, laminitis, epaxial muscle wastage or muscle atrophy, weight loss, abnormal fat distribution, polydipsia, polyuria, bacterial infections, intestinal parasitism and lethargy or depression (Ireland and McGowan 2018; Fortin et al. 2021; Tatum et al. 2021). Therefore, horses with PPID tend to have higher helminth faecal egg counts and may need a more aggressive parasite prevention program, leading to a greater difficulty in keeping their body condition and to a higher cost (McFarlane et al. 2010; Horn et al. 2019). Still, owner-reported prevalence is lower than that of the pooled veterinary-reported prevalence (Ireland and McGowan 2018). Many of these clinical signs of PPID are readily recognized by owners but are mistakenly considered as signs of old age (Mcgowan et al. 2013; Horn et al. 2019). As pointed out before(Ireland and McGowan 2018), there is an imperative need to educate owners to identify clinical signs of PPID well enough for them to seek veterinary help as early as possible in the disease progression.

2. Research objectives

It has been suggested that horses with PPID are more likely to have higher helminth faecal egg counts than healthy horses and may need a more aggressive parasite prevention program (McFarlane et al. 2010; Horn et al. 2019). Since there aren't many studies evaluating the effect of pergolide in immunosuppression (Kirkwood et al. 2022) and its effects on a potential decrease in hFEC, this pilot study in-tended to evaluate the effect of the oral administration of this drug in the reduction of hFEC and time to egg reappearance, as

compared to no treatment, over a period of 6 months, in 15 privately owned horses ≥ 15 years old diagnosed with PPID in non-autumn months (plasma ACTH >29 µg /ml) by first opinion veterinarians. Furthermore, the main clinical signs to be addressed in owner education regarding PPID early recognition were identified by means of an owner questionnaire and the owner's medication compliance was investigated in order to better advice on overcoming barriers.

At present, we intended to carry out a pilot study to compare a standard treatment (pergolide) with no treatment, focusing on a potential further benefit of pergolide therapy vs. parasite burden reduction. Furthermore, the PPID questionnaires aimed to increase owner and veterinarians' awareness of this condition in order to favour early diagnosis and treatment. The effort to maintain compliance with the treatment for the length of six months aimed at creating a habit of treating these horses and reaching the time point where true positive changes can actually be acknowledged by both owners and veterinarians.

3. Materials and Methods

The primary outcomes (dependant variables) of the present study were hFEC and time to egg reappearance. The secondary outcomes to be evaluated were physical exam (including weight, body condition and a cresty neck scores), a PPID questionnaire to be answered by the owners and a longitudinal compliance record for the researchers.

3.1. Horses

This study involved 15 privately owned horses, ≥ 15 years of age, from first opinion practice, as it has the potential of being more representative of the target population. Convenience samples recruited via hospital or referral practices are likely to be biased towards cases with more advanced stages of PPID or with concurrent diseases, not being so readily generalizable to the general population.

The inclusion criteria were a basal ACTH plasma concentration >29 µg/ml, as an objective measure, and a PPID phenotype characterized by hypertrichosis, delayed seasonal haircoat shedding, distended abdomen, regional adiposity (periorbital; cresty neck), skeletal muscle atrophy, decreased athletic performance and lethargy.

These horses were evaluated by first opinion veterinarians in non-Autumn months and were included in the study from December 2021 to July 2022.

They were then divided in two groups, a treatment group (pergolide) with 10 horses, and a control group (no treatment) with 5 horses. Since it is not ethical to withhold an authorized standard treatment (in this case pergolide), only horses whose owners did not agree to carry out this treatment were included in the control group. Therefore, horses were allocated to either group depending on whether the owners accepted treating them with pergolide or not.

Horses were excluded if suffering from clinically detectable pain or concurrent systemic disease, or if they were overtly anxious or unsettled in their environment.

3.1.1. Clinical exam

Age and gender were registered for all horses. The presence of PPID related clinical signs was evaluated, such as hypertrichosis, delayed seasonal haircoat shedding, distended abdomen, regional adipose accumulation (periorbital; cresty neck), musculoskeletal atrophy, decreased athletic performance and lethargy.

Body condition score was attributed according to the William Martin-Rosset's grading system in a 0-to-5 scale (Martin-Rosset 2015). A cresty neck score was also applied, following the Carter et al (2009) (Carter et al. 2009) grading system, in a 0-to-5 scale.

Furthermore, the weight was measured with horse weight tapes considering the girth diameter (the mean value of three different tapes was used).

3.1.2. Housing

The type of housing was registered for every horse as stabled with other animals, always in a paddock or mixed.

3.2. Questionnaire

A questionnaire was filled by the owners in order to obtain more specific information about the management of the horses. It included questions regarding the horses' stabling routines, general management, work schedule and the occurrence of clinical signs which are usually associated with PPID.

Furthermore, an informed consent form was signed by the owners, which included pertinent information regarding PPID in order to raise more awareness of the disease as to its risk factors, clinical signs and management (Table 1).

Table 1- Keypoints included in the informed consent form signed by the owners

Parameter	Keypoints
What is Pituitary Pars Intermedia Syndrome?	It is one of the most common endocrine diseases in horses, especially older ones. It results from an adenoma of the pituitary gland that leads to an overproduction of adenocorticotropic hormone (ACTH)
Risk Factors	Horses older than 15 years of age
Clinical Signs	Presence of delayed seasonal haircoat shedding Hypertrichosis Distended abdomen Regional adipose accumulation (periorbital; cresty neck) Musculoskeletal atrophy (eg. epaxial muscle wastage or "sway back") Weight loss Decreased athletic performance Lethargy
Treatment	Oral administration of Pergolide, 0.002 mg/kg per day (1mg/day for a 500 kg horse)

Long-term care	Clipping during summer Adequate feeding management (senior extruded pellet diet and a mixture of alfalfa and oat chaff) Regular vaccination Dental work done each 3-6 months Faecal egg count each 3 months in order to deworm in a timely manner Regular hoof trimming
Complications of this disease	Laminitis Difficulty in controlling infections Recurrent colic
Premisses of the study	Horses with PPID might have higher faecal egg counts, which leads to difficulty in maintaining body condition and a more frequent deworming program that results in higher costs for the owners; Owners may not be able to identify PPID clinical signs in a timely manner and may not be aware of the importance of treatment compliance.
Research's main goals	This study intends to evaluate the effect of the oral administration of Pergolide in the reduction of helminth faecal egg count and time to egg reappearance, as compared to no treatment, over a period of 6 months, as well as to increasing owner awareness of the disease and of its treatment.

3.3. Non-invasive blood pressure measurement

The systolic and diastolic arterial blood pressures (mmHg) were measured using a Kompernass Balance KH8099® electronic sphygmomanometer applied to the tail to evaluate the middle coccygeal artery, always ensuring the correct posture of the horse, since lowering the head significantly lowers systolic and diastolic arterial blood pressure (Bailey et al. 2008; Söder et al. 2012; Alberti et al. 2019) (Figure 3). Triplicate measurements were collected to ensure the precision of the results and the mean value was used for further analysis.

Regarding systolic blood pressure, the normal values ranges between 80 to 144 mmHg, while diastolic blood pressure ranges from 49 to 105 mmHg (Parry et al. 1983; Söder et al. 2012).

Figure 3- Non-invasive blood pressure measurement using an electronic sphygmomanometer applied to the tail

3.4. Blood sample collection for basal plasma ACTH concentration

Blood samples were collected from the jugular vein during non-fall months (December to July) into vacuum 10 ml tubes containing potassium-EDTA. Considering that stress can alter the ACTH values, care was taken to collect the samples in a calm and quiet environment.

The blood collection occurred in three occasions:

- On day one, to obtain the basal ACTH values of every horse;
- At two months, to establish the possible need for pergolide dose adjustment;
- At six months, to evaluate the effects of the six months' pergolide treatment.

All the blood samples for plasma ACTH concentration measurement were sent to a reference laboratory refrigerated at 4ºC, within three hours of collection (McGowan et al. 2013; Hart et al. 2020; Hinrichsen et al. 2022; Kirkwood et al. 2022). Evaluation of plasma ACTH concentration was performed using a Siemens Immulite 2000 equipment for an enzymatic immunoassay with chemiluminescent reading (Banse et al. 2018).

3.5. Faecal sample collection and helminth faecal egg count (hFEC)

Fresh faecal samples were collected on days 1, 30, 60, 90, 120, 150 and 180 for hFEC.

Whenever fresh stools were available, faecal samples were collected directly from the horses' stable, otherwise collection was carried out by rectal palpation.

The samples were analysed using the Mini-Flotac (Cringoli et al. 2017) and the McMaster methods (Dias de Castro et al. 2017). In both techniques, eggs per gram (EPG) values above 200 were considered significant (Nielsen 2022)]. After the first evaluation, all horses from both groups were dewormed with Ivermectin 0,2mg/kg (Eqvalan® - oral paste for horses) to allow an equalitarian evaluation of subsequent hFEC.

3.6. Pergolide administration

Pergolide was started at the recommended dose of 0.002 mg/kg per day (1mg/day for a 500 kg horse). After 8 weeks of treatment, if neither a considerable improvement in the laboratory test results (plasma ACTH), nor an evidence of clinical improvement were observed, the dose was increased by 0.001 mg/kg increments monthly until improvement was achieved (Schott 2002; Hart et al. 2020).

An initial side effect of this treatment may be inappetence, where the dose can be halved for 1-2 weeks and then gradually increased. Depression and diarrhea may also occur, as well as excessive perspiration, in which case the drug should be discontinued for 2-3 days and then recommenced at half dose, followed by 0.5 mg increments every 2-4 weeks. Cardiac side effects have not been recognized in horses (Spelta and Axon 2012; McGowan et al. 2013; Jacobson et al. 2014; Schwarz and Ihry 2020).

In this study, Pergoquin 1mg per tablet® from Richter Pharma was administrated orally for six months.

3.7. Compliance with pergolide treatment

Owner compliance with pergolide treatment recommendations was considered to be a problem due to the need for long term treatment and this may be a potential source of bias.

Therefore, the researchers did an initial in-person visit to each horse, where they carried out a conversation with the owner and other carers (e.g. groom, rider) in order to hand out an informative leaflet which included the information in Table 1, for further reference. This information was carefully discussed when the informed consent was signed.

Furthermore, monthly in-person visits were carried out by the researchers in order to supervise and register owner/carer compliance. Any existing doubts were clarified during these visits.

3.8. Statistical analysis

Statistical analysis was carried out using the IBM® SPSS® V.8. program and different tests were used to evaluate all the parameters included in the study.

To compare means of 2 independent samples (treatment group and control group) concerning the quantitative dependent variables (ACTH value, hFEC, weight and cresty neck score), the parametric T test for independent samples was used. As a non-parametric alternative the Mann-Whitney U test was used.

To analyse between subjects, the Wilcoxon test was used as a non-parametric alternative to the T-test for 2 paired samples. For the intra-subject analysis with more than 2 paired measures, the parametric test ANOVA repeated measures was used, with the multiple comparison Fisher's least significant difference test (LSD). As a non-parametric alternative, the Friedman test was used, with multiple Dunn-Bonferroni comparisons.

At the non-parametric level, the chi-square test was also used to analyse the association/independence between qualitative variables (body condition score). For this purpose, the assumptions for its use were ensured, namely the absence of expected frequencies below 1 and less than 20% of cells with expected frequencies below 5. In case these conditions could not be met, the statistical significance of the Fisher's exact test was used. In order to evaluate trends of significant associations between categories, the criterion of adjusted residuals >|1.96| was used. For all tests the significance level was $p<0.05$.

4. Results

4.1. Clinical exam

4.1.1. Age

Age mean and standard deviation (SD) was 24.1 ± 4.134 years in the treatment group and 31 ± 4.147 in the control group. Age distribution in both groups can be seen in table 2.

Table 2- Age distribution in the treatment and control groups

	Age	Frequency
Treatment Group	19	10%
	21	30%
	22	10%
	23	10%
	24	10%
	29	20%
	32	10%
Control Group	28	40%
	29	20%
	31	20%
	39	20%

4.1.2. Gender

The treatment group consisted on 7 geldings (n= 7/10) and 3 mares (n= 3/10). The control group included 5 geldings (n= 5/5).

4.1.3. Body condition scores

The monthly body condition scores in the control and treatment groups throughout the 6 months of the study are shown in figure 4. Results at day 120 are not included due to the lack of records for the control group and only 50% of records for the treatment group.

Scores of 1 and 2 indicated a low body condition and scores of 4 and 5 indicated a high body condition. A score of 3 was considered to be an ideal body condition.

In the control group, at the beginning of the study 20% of the horses were very underweight, 20% were underweight and a majority of 60% had an ideal weight. As for the horses in the treatment group, 10% were very underweight, 20% were underweight, 20% had an ideal weight, 40% were overweight and 10% were highly overweight (Figure 4).

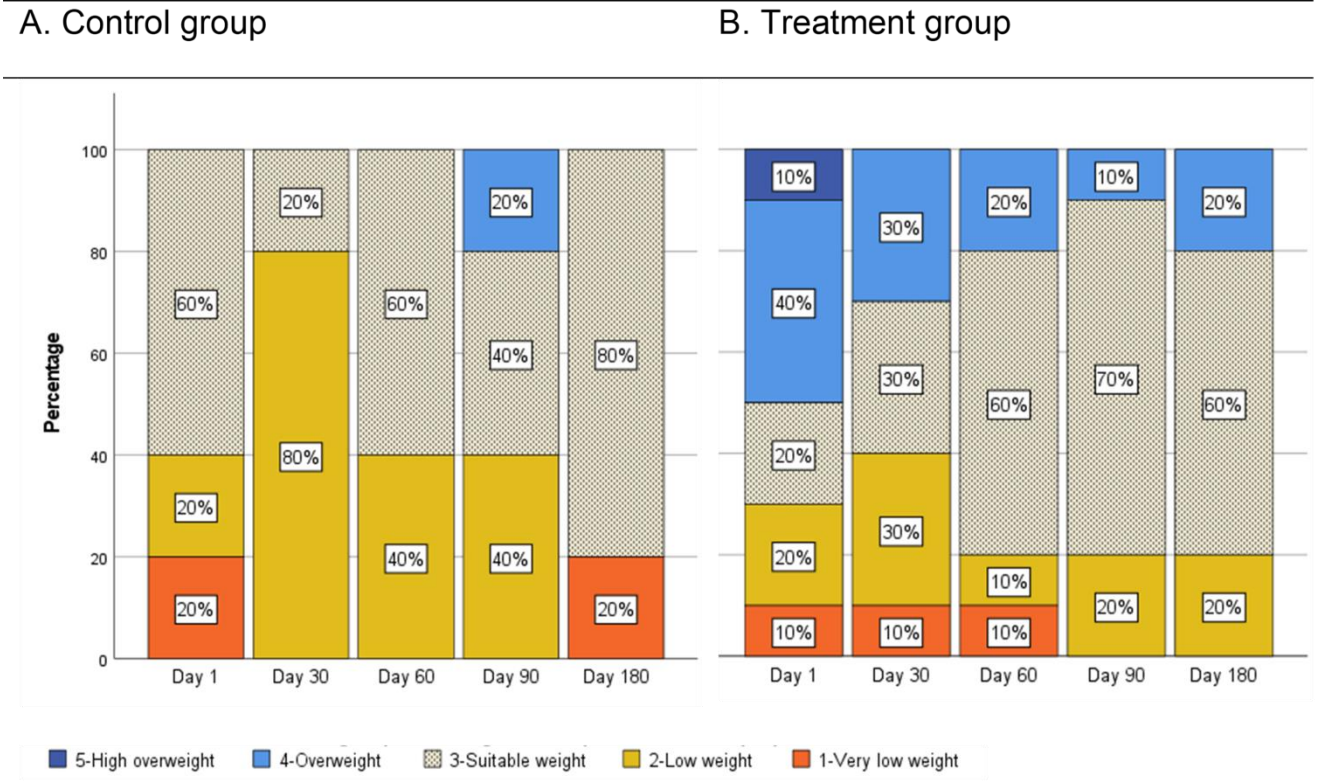


Figure 4- Body condition scores per group throughout the 6 months according to the William Martin-Rosset's (Martin-Rosset 2015) **grading system in a 0-to-5 scale**

The difference in the proportion of body condition scores throughout the evaluation moments, for the control and treatment groups is shown in table 3.

Table 3- Proportion of body condition scores throughout the evaluation moments.

Group	Evaluation periods	Body Condition Scores (1-5) 1-2 Underweight	3 Ideal weight	4-5 Overweight	X^2	p	Adjusted Residuals
Control	Day 1	40%	60%[1]	0%[3]	71.429	<0.001	5.8[1] -5.8[2]
Treatment	Day 1	30%	20%[2]	50%[4]			-8.2[3] 8.2[4]
Control	Day 1	40%	60%	0%[6]	163.636	<0.001	8.1[1] -5.4[2]
	Day 30	80%[1]	20%[3]	0%[6]			-7.2[3]-2.7[4]
	Day 60	40%	60%	0%[6]			6.3[5] -2.3[6]
	Day 90	40%	40%[4]	20%[7]			9.1[7]
	Day 180	20%[2]	80%[5]	0%[6]			
Treatment	Day 1	30%	20%[2]	50%[6]	86.859	<0.001	3.61[1]-6.3[2]
	Day 30	40%[1]	30%[3]	30%			-4.0[3] 2.7[4]
	Day 60	20%	60%[4]	20%			4.9[5] 6.1[6]
	Day 90	20%	70%[5]	10%[7]			-4.1[7]
	Day 180	20%	60%[4]	20%			

When comparing the control and treatment groups at the beginning of the study, the body conditions show significant differences ($X^2(2)$) = 71.429; p<0.001). The control group presents a higher proportion of horses with an ideal weight (60% vs. 20% ResAdjust=5.8), while the treatment group has a significantly higher proportion of overweighed horses (50% vs. 0% ResAdjust= 8.2).

Regarding the evolution of the body condition of the horses in the control group, it can be observed that on day 30, 80% of the horses were underweight and only 20% had an ideal

weight. While on day 60 only 40% were underweight and 60% had already reached an ideal weight. On day 90, the proportion of underweight horses continued to be 40%, but 20% of the horses became overweight. At the end of the study, the highest proportion of horses with an ideal weight is observed, but 20% of the horses still show a very low weight.

Therefore, the control group results presented in Table 3 reveal significant differences in the body condition of the horses throughout the study ($X^2(8)=163.636$; $p<0.001$). The proportion of underweight horses on day 30 is significantly higher (80%, ResAdjust= 8.1), and on day 180 it is significantly lower (20%, ResAdjust= -5.4). On the other hand, the proportion of horses with ideal weight is significantly lower on day 30 (20%, ResAdjust= -7.2) and day 90 (40%, ResAdjust= -2.7) and significantly higher on day 180 (80%, ResAdjust= 6.3). The highest proportion of overweight horses (20%, ResAdjust= 9.1) is observed on day 90.

Regarding the treatment group, the results depicted in Table 3 show that, on day 30, the proportion of underweight horses increases to 30%, and those with excess weight decrease to 30%. On day 60, the proportion of horses with an ideal weight increased to 60% and the under and overweight horses decreased by 10% and 20%, respectively. On day 90, the proportion of horses with an ideal weight reached the highest percentage of 70%, subsequently decreasing to 60% on day 180, with the remaining 40% of horses divided between the underweight and overweight categories.

A significant difference in the body condition scores of the treatment group horses throughout the study ($X^2(8)=86.859$; $p<0.001$) can also be seen in Table 3 . The proportion of underweight horses at day 30 is significantly higher than that recorded in the remaining evaluation periods (40%, ResAdjust= 3.6). On day 60 (60%, ResAdjust= 2.7), day 90 (70%, ResAdjust= 4.9) and day 180 (60%, ResAdjust= 2.7), the proportion of horses with an ideal body condition is significantly higher than that recorded on days 1 and 30. Finally, the proportion of overweight horses at the beginning of the study (50%, ResAdjust= 6.1) is significantly higher than that recorded in the remaining evaluation moments. This proportion of over-weight horses is most significantly lower on day 90 (10%, ResAdjust= -4.1).

4.1.4. Cresty neck scores

Table 4 presents the results of cresty neck scores from both control and treatment groups from day 1 to day 180.

Table 4- Cresty neck scores of control and treatment groups

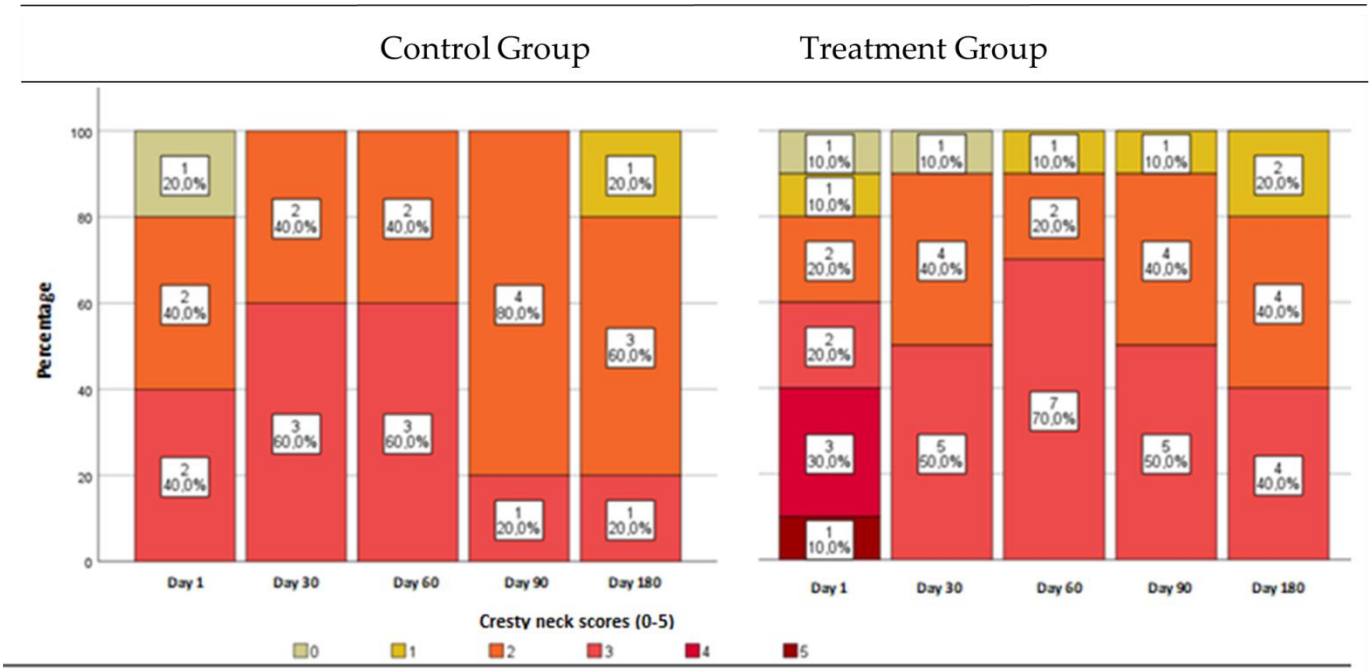
Group	Evaluation Period	Cresty neck scores (0-5)		$Fr_{(4)}$ $t_{(13)}$	p
		Min.-Max.	MR or Median ± SD		
Control	Day 1	0-3	2.00±1.22	-1.002	0.334
Treatment	Day 1	0-5	2.80±1.55		
Control (n=5)	Day 1	0-3	2.60	4.526	0.339
	Day 30	2-3	3.70		
	Day 60	2-3	3.70		
	Day 90	2-3	2.70		
	Day 180	1-3	2.30		
Treatment (n=10)	Day 1	0-5	3.65	7.167	0.127
	Day 30	0-3	2.65		
	Day 60	1-3	3.40		
	Day 90	1-3	2.90		
	Day 180	1-3	2.40		

Legend: MR- Mean Rank, SD- Standard Deviation

No significant differences in the cresty neck score were found between groups on day 1 ($t(13)= -1.001$; $p= 0.334$) as this score was 2.80 ± 1.55 in the treatment group and 2.00 ± 1.22 in the control group. Hence, suggesting that the adipose accumulation on the neck at baseline was the same for both the control and the treatment group horses.

On day 1 the horses from the control group had cresty neck scores ranging from 0 to 3, with only one horse having a score of 0, 60% had a score of 3 and 40% had a score of 2 (MR= 2.60). On days 30 (MR= 3.70) and 60 (MR= 3.70), 60% of the horses have a score of 4 and 40% a score of 3. On day 90 (MR= 2.70) only 20% had a score of 3, with 80% scoring 2. On day 180, 20% of the horses continued to show score 3, there was a decrease to 60% of horses with score 2 and a decrease to 20% of horses with score 1 (MR= 2.30) (Figure 5). Despite a decrease in the scores on day 60, the differences observed between the evaluation moments are not statistically significant ($Fr(4)= 4.526$; $p= 0.334$), therefore the adipose accumulation in the horses' neck of the control group did not change significantly over the course of the study.

As to the treatment group, on day 1 the horses had cresty neck scores ranging between 0 and 5, with 10% of the horses having a score of 5, 30% a score of 4, 20% a score of 3 or 2 and 10% a score of 1 or 0 (MR= 3.65). On day 30, no horses had a score of 4 and 5 and there was an increase to 50% in the proportion of horses with a score of 3, as well as an increase to 40% in horses with a score of 2 (MR= 2.65). On day 90 (MR= 2.90) there was a rise to 70% in the proportion of horses with score 3, but on day 180 this proportion decreased to 40%. The remaining horses on day 180 (MR= 2.40) had scores of 2 or 1, corresponding to 40 % and 20 % respectively. However, the differences observed were not



statistically significant (Fr(4)=7.167; p=0.127), thus accumulation of fat on horses' necks did not change significantly over the course of the study in the treatment group either.

Figure 5- Cresty neck scores, following the Carter et al (2009)(Carter et al. 2009) **grading system, in a 0-to-5 scale**

4.1.5. Horses' Weight

Table 5 and Figure 6 present the treatment and control groups horses' average weight which was measured once a month for 6 months. At the beginning of the study, the 5 horses from the control group showed an average weight of 458 ± 37.1 kg and the 10 horses from the treatment group 462.3 ± 43.4 kg. Therefore, at day 1, the baseline mean values for the weight were not statistically different between groups (t(13)=-0.185; p=0.858), showing that the horses from both groups had overall similar weights.

Table 5- Horse weight per group throughout the evaluation moments

Evaluation periods	Group: Control n	Control M ± SD	Treatment n	Treatment M ± SD	$t_{(13)}$	p
Day 1	5	458.4^{12} ± 37.1	10	462.6 ± 43.4	-0.185	0.856
Day 30	5	461.4^{34} ± 40.8	10	458.8 ± 46.3		
Day 60	5	455.6 ± 34.1	10	466.4^{12} ± 43.9		
Day 90	5	447.4^{13} ± 33.4	10	456.6^{1} ± 39.9		
Day 120			5	465.8 ± 65.8		
Day 180	5	$439,4^{24}$ ± 33.0	10	451.3^{2} ± 39.6		
LSD multiple comparisions	$F_{(4, 16)}$=6.171; *p=0.003* *p=0.015*[1] *p=0.032*[2] *p=0.034*[3] *p=0.041*[4]		$F_{(4, 36)}$ =2.917; p=*0.035* *p=0.028*[1] *p=0.002*[2]			

Legend: M- Mean, SD- Standard Deviation, Superscript 1,2,3,4- Equal numbers refer to mean differences with respective p values

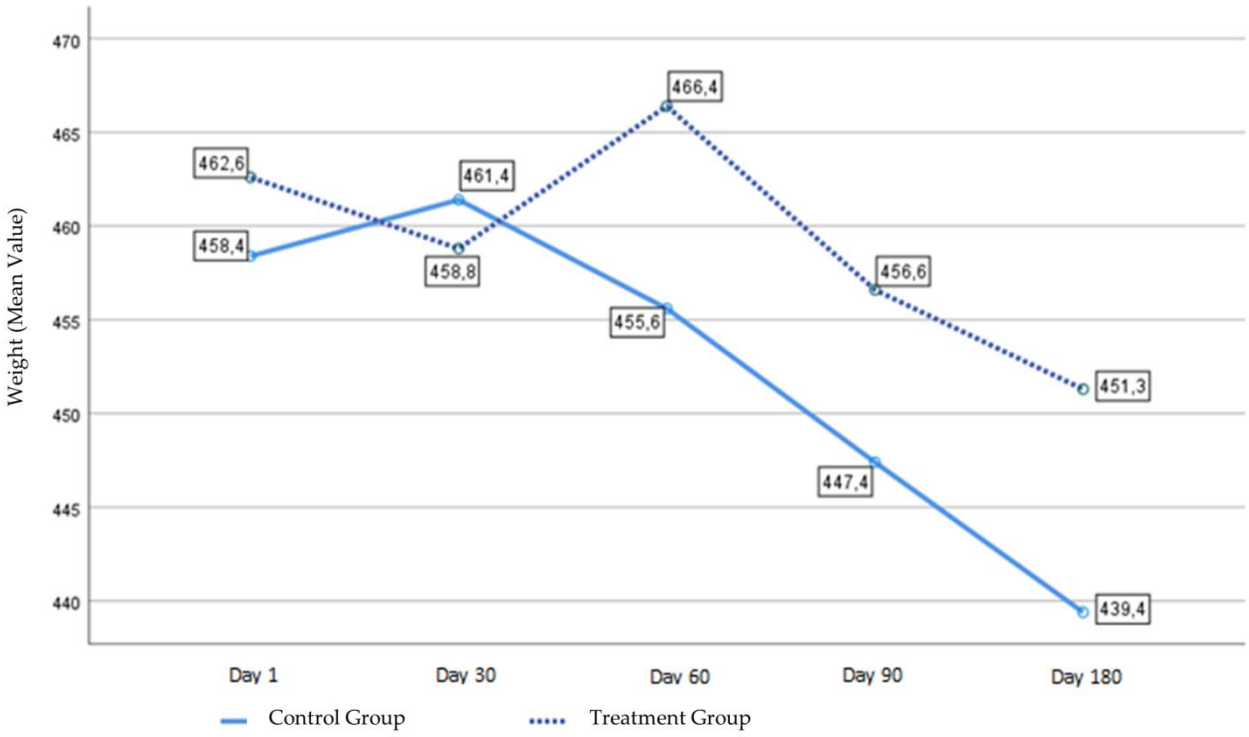


Figure 6- Mean weight of control and treatment group horses over the study assessment periods

The results show a slight increase in the average weight of the control group in the first month (M= 461.4; SD= 40.8) followed by a gradual decrease until the end of the study, where the average weight is 439.4 Kg (SD= 33.0).

These results reveal that there are statistically significant differences in the control group horses' weight over time (F(4,16)= 6.171; p= 0.003). Thus, there is a significant weight loss on day 90 (M= 447.4; SD= 33.4), compared to day 1 (M= 458.4; SD= 37.1; p= 0.015), and day 30 (M= 461.4; SD= 40.8; p= 0.034). There is also a significant decrease in weight on day 180 (M= 439.4; SD= 33.0) compared to day 1 (M= 458.4; SD= 37.1; p= 0.032) and day 30 (M= 461.4; SD= 40.8; p= 0.041).

In summary, the horses show a significant weight decrease on days 90 and 180 compared to days 1 and 30.

Regarding the treatment group, the highest weight was recorded on day 60 (M= 466.4; SD=43.9) and the lowest weight on day 180 (M= 451.3; SD= 39.6). On day 120, only 5 of the horses' weight were recorded, so this evaluation period will not be considered. The results obtained reveal that there are also statistically significant differences in the weight of the horses in the treatment group over time (F(4,36)=2.917; p= 0.035). Thus, there is a significant weight loss on day 90 (M= 456.6; SD= 39.9; p= 0.028) and on day 180 (M= 451.3; SD= 39.6; p= 0.002) compared to the weight on day 60 (M= 466.4; SD= 43.9).

In conclusion, even though there was a significant weight loss of the horses in the treatment group in the final phase of the study (day 90 and 180) compared to day 60, these horses always kept their weight above control group horses from day 60 onwards.

Furthermore, the decrease in average weight from day 90 to day 180 was not as sharp as for the control group horses.

4.2. Housing

There were different types of housing situations. In the treatment group, 4 out of the 10 horses lived in stables with other horses, 5 were in paddocks during the day and in a stable with other horses at night, and 1 lived in a stable with goats. In the control group all 5 horses lived in a stable with other horses.

4.3. Questionnaires

The results of the owners' questionnaires are presented in table 6.

Table 6- Results of the owners' questionnaires (days 1 and 180) for the control and treatment groups

	Control Group (N=5)								Treatment Group (N=10)							
	Day 1				Day 180				Day 1				Day 180			
Question	No		Yes		Didn't improve		Improved		No		Yes		Didn't improve		Improved	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
1. Does the horse have longer/curly/dull haircoat?	0	0.0	5	100	0	0.0	5	100	0	0.0	10	100	1	10.0	9	90.0
2. Does the longer haircoat shed during the summer?	5	100	0	0.0	---		5	100	5	50.0	5	50.0	1	11.1	8	88.9
3. Did the horse has laminitis?	5	100	0	0.0	---		---		9	90.0	1	10.0	1	50.0	0	0.0
4. Is the horse occasionally lame?	5	100	0	0.0	---		---		9	90.0	1	10.0	1	33.3	2	66.7
5. During the stride, does the fetlock lower towards the ground? (desmitis/laxity of the suspensory ligament)	3	60.0	2	40.0	1	100	0	0.0	9	90.0	1	10.0	0	0.0	1	100
6. Does the horse appear sweaty in specific zones or in general?	5	100	0	0.0	---		---		8	80.0	2	20.0	1	33.3	2	66.6
7. Does the horse drink more water than normal?	5	100	0	0.0	---		---		8	80.0	2	20.0	1	33.3	2	66.6
8. Does the horse urinate more than normal? (Bedding appears wetter)	5	100	0	0.0	---		5	100	4	40.0	6	60.0	0	0.0	6	100

9. Does the horse eat more than normal?	0	0.0	5	100	0	0.0	5	100	4	40.0	6	60.0	1	16.7	5	83.3
10. Are there any specific fat deposits? (cresty neck, base of the tail,supraorbital)	1	20.0	4	80.0	2	50.0	2	50.0	3	30.0	7	70.0	3	42.9	4	57.1
11. Does the horse's belly look distended (abdomen with a pendular look)?	0	0.0	5	100	0	0.0	5	100	0	0.0	10	100	1	12.5	7	87.5
12. Has the horse lost muscle mass? (neck, back and croup)	1	20.0	4	80.0	2	40.0	3	60.0	7	70.0	3	30.0	2	25.0	6	75.0
13. Has the horse had any episode of colic?	5	100	0	0,0	---		---		7	70,0	3	30,0			2	100
14. Does the horse have less energy (lethargy)?	3	60.0	2	40.0	3	75.0	1	25.0	5	50.0	5	50.0	2	28.6	5	71.4
15. Does the horse fall asleep standing and fall? (narcolepsy)	5	100	0	0.0	---		---		8	80.0	2	20.0				
16. Has the horse had any wound that was difficult to heal?	5	100	0	0.0	---		---		9	90.0	1	10.0	---		1	100
17. Has the horse had any other infection that was difficult to treat?	5	100	0	0.0	---		---		6	60.0	4	40.0				
18. Does the horse frequently have parasites?	5	100	0	0.0	---		---		10	100	0	0,0	---		---	
19. Has the horse been dewormed?	0	0.0	5	100	---		---		0	0.0	10	100	---		---	
20. Does the horse have any eyeshight problem? (dry eye / recurrent corneal ulcer / blindness)	4	80.0	1	20.0					9	90.0	1	10.0	---		1	100
21. Does the horse have any neurological problem? (seizure)	5	100	0	0.0	---		---		10	100	0	0.0	---		---	

4.4. Blood pressure

On day 1, when comparing the mean value of the systolic pressure measurements obtained in the control group with the mean value of the treatment group, using Mann-Whitney U test for independent samples, there were no statistically significant differences (p= 0.679). The same was true for the systolic pressure on day 180 (p= 0.371). Likewise, there were no statistically significant differences for the diastolic pressure between groups on day 1 (p= 0.254) and on day 180 (p= 0.594).

Table 7 presents the results of systolic and diastolic blood pressure on days 1 and 180 for each of the study groups. It can be seen that in both control and treatment groups, on day 180 the blood pressures (systolic and diastolic) show higher results. However, the observed differences are not statistically significant (p>0.05), therefore there is no evidence that systolic and diastolic blood pressure increase on day 180, with or without treatment.

Table 7- Systolic and diastolic blood pressure comparison between day 1 and 180 for both groups

Arterial Pressure	Group	N	Day 1 M ± SD	Day 180 M ± SD	T(4)(9)	p
Systolic	Control	5	131.40 ± 15.77	145.40 ± 18.90	-1.929	0.126
	Treatment	10	125.90 ± 18.69	135.50 ± 21.59	-0.936	0.374
Diastolic	Control	5	83.80 ± 10.57	89.80 ± 5.76	-1.007	0.371
	Treatment	10	77.80 ± 8.20	85.10 ± 11.21	-1.623	0.139

4.5. ACTH

Table 8 and Figure 7 show the ACTH serum results for the control group and the treatment group at 3 time points, baseline, day 60 and day 180. ACTH values were not evaluated in the control group on day 60 because there was no treatment to be adjusted. Hence, the analyses carried out for the control group sample were focused on the beginning (day 1) and the end of the study (day 180).

Table 8- ACTH changes (µg/L) for the control and treatment groups

Group	ACTH	N	MR or M±SD	Statistic	p
Control	Day 1	*5*	*6.40*[MR]	17.000[a]	0.327
Treatment	Day 1	*10*	*8.80*[MR]		
Control	Day 1 higher than Day 180	5	3.00[MR]	-2.023[b]	0.043
(n=5)	Day 180 higher than Day 1	0	0.00[MR]		
Treatment	Day 1		[1,2]74.4±32.9	24.405[c]	<0.001
(n=10)	Day 60		[1]28.8±10.2		[1,2]0.002
	Day 180		[2]24.0±6.4		

Legend: M- Mean, SD- Standard Deviation, MR- Mean Rank, a- Mann-Whitney Test U Statistics, b- Wilcoxon Z-Test for Paired Samples, c- ANOVA test F-statistic Repeated measures, 1 and 2 significance of LSD Multiple

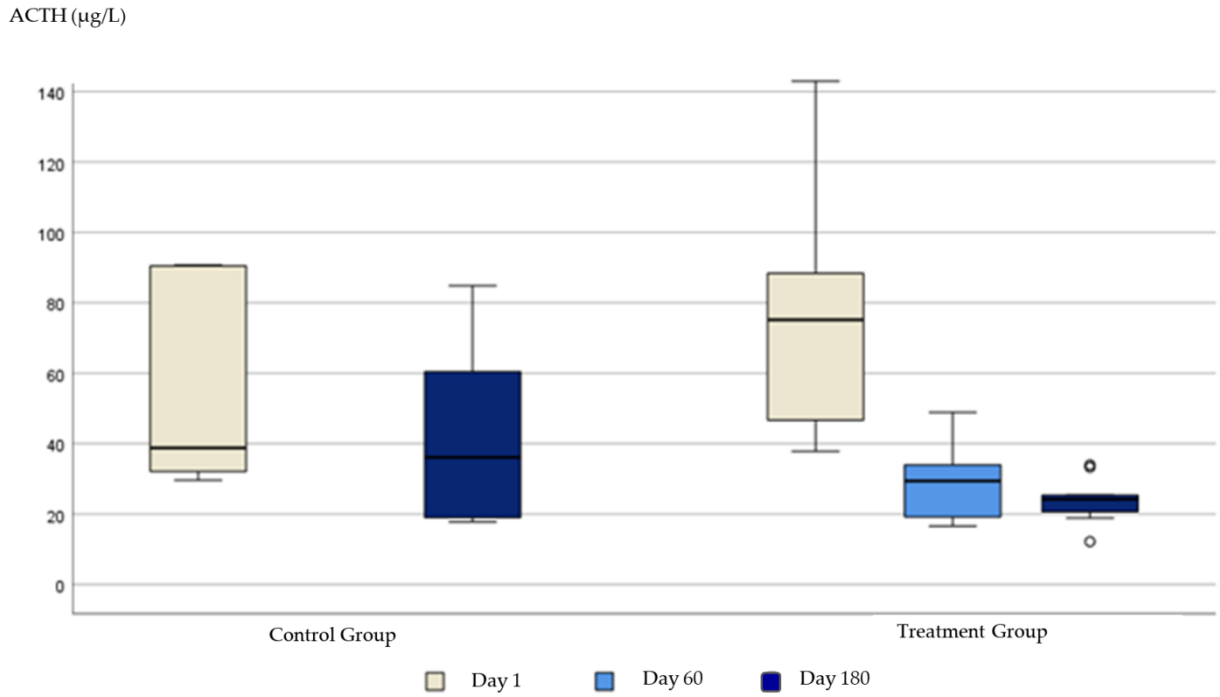


Figure 7- ACTH serum values for the control and treatment group horses over time

When comparing the ACTH results obtained on day 1 for the control group horses (MR= 6.40) and for the horses treated with pergolide (MR= 8.80), no statistically significant differences were found (U= 17.000; p= 0.327). Therefore, the horses in both groups had similar ACTH values at baseline (Table 8).

The pergolide treatment group horses had a mean baseline ACTH of 74.4 µg/L (SD= 32.9), on day 60 the mean dropped to 28.8 µg/L (SD= 10.2) and on day 180 to 24.0 µg/L (SD= 6.4). These average values were statistically significantly different (F(1,119 10,787)=24.405; p<0.001) with the mean ACTH of horses on days 60 and 180 being significantly lower than ACTH at baseline (LSD p=0.002).

A significant reduction in average ACTH values from day 1 to day 180 was also observed for the horses in the control group (MR= 0.00; Z= -2.023; p= 0.043), although ACTH values on day 180 in this group showed a much wider distribution and included much higher values (Figure 7).

3.6. Helminth Faecal Egg Count (hFEC)

3.6.1. hFEC using the Mini-Flotac technique

Table 9 and Figure 8 show the results of the hFEC of the control group and the treatment group over the 5 time points of the study (days 1, 30, 60, 90 and 180), using the Mini-Flotac technique.

In the control group, on day 1, 60% of the horses had an egg count of zero which then increased to 80% after 90 days. Nevertheless, only 20% of the horses had clinically relevant counts (≥ 200EPG), with an average of 955 EPG on day 1, 480 EPG on day 30, 655 EPG on day 60 and 220 EPG on day 180. On day 90 no horse showed clinically relevant values of EPG (0%).

In the treatment group, on day 1, 60% of the horses had an egg count of zero as well, but the same was reported for day 60. On day 30, 90% of horses showed an egg count of zero and on days 90 and 180, 80% of horses maintained an egg count of zero. Only 10% of horses in this group showed counts with relevant clinical values on day 1 (600 EPG) and 20% on day 60 (415 and 1150 EPG). On days 30, 90 and 180, no horses in the treatment group showed relevant clinical values of EPG.

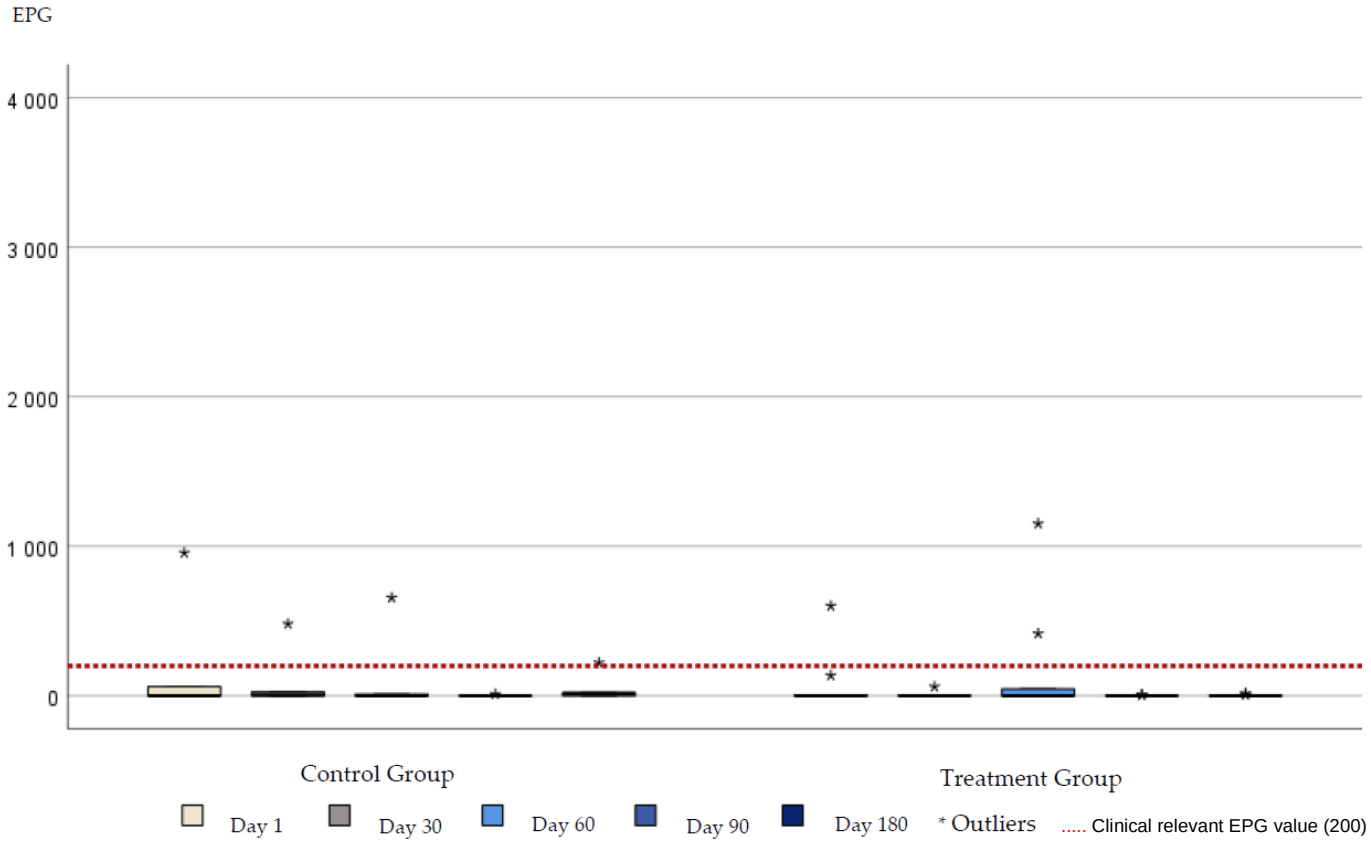


Figure 8- Helminth faecal egg counts for the control and treatment groups, using the Mini-Flotac technique

When comparing EPG values on day 1 from the control group (MR=8.40) and the treatment group (MR= 7.80), no significant differences were observed (U= 23.000; p= 0.859). As a result, it can be concluded that EPG baseline values were homogenous among the control and treatment group horses (Table 9).

Despite there being slight variations in EPG counts for the control group throughout the 5 evaluation moments of the study, there were no statistically significant differences (Fr(4)=2.915; p= 0.572).

Regarding the treatment group there were also no statistically significant differences in EPG (Fr(4)=3.919; p= 0.417) throughout the study when using this technique.

3.6.2. hFEC using the McMaster technique

Table 9 and Figure 9 show the results of the hEFC for the control and the treatment groups, over the 5 time points of the study, using the McMaster technique.

On day 1, no statistically significant differences were seen (U= 16.000; p= 0.189) in EPG between the control group (MR= 9.80) and the treatment group (MR= 7.10) (table 10). This suggests that there was homogeneity of EPG values amongst groups at baseline.

Regarding the control group, over the 5 evaluation periods, between 40 to 80% of the horses had 0 EPG. EPG values were lower on days 1 and 180 and higher on days 60 and 90. Furthermore, on day 1, 40% of the horses had clinically relevant counts (≥ 200 EPG) of 350 and 2050 EPG. On days 30, 60 and 180, 20% of the horses also had clinically relevant counts of 1300, 1800 and 300 respectively. Only on day 90 no horse showed clinically relevant values.

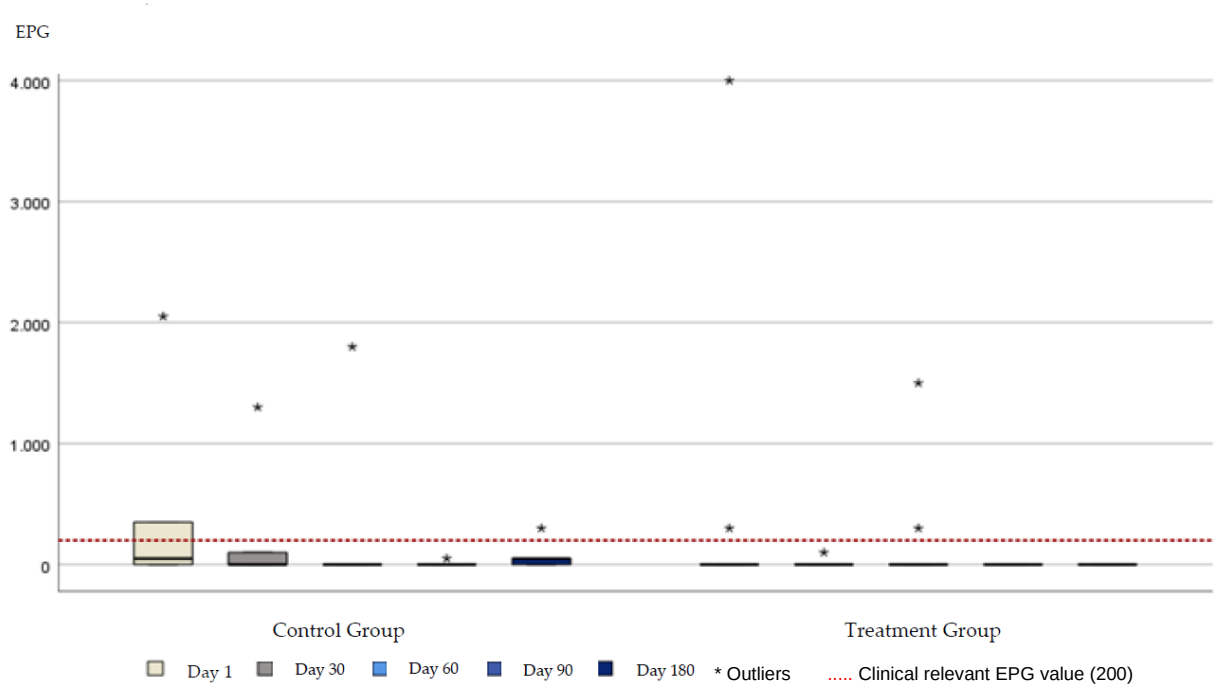


Figure 9- Helminth faecal egg counts for the control and treatment groups, using the McMaster technique

Table 9- Helminth faecal egg counts using the MiniFlotac and the McMaster techniques

Technique	Group	Evaluation	Day 1		Day 30		Day 60		Day 90		Day 180	
			n	*%*	n	*%*	n	*%*	n	*%*	n	*%*
MiniFlotac	Control n=5	0	3	*60*	2	*40*	3	*60*	4	*80*	2	*40*
		<200	1	*20*	2	*40*	1	*20*	1	*20*	2	*40*
		≥200	1[a]	*20*	1[a]	*20*	1[a]	*20*	-	---	1[a]	*20*
		Value[a]		*955*		*480*		*655*	-	---		*220*
	Treatment n=10	0	6	*60*	9	*90*	6	*60*	8	*80*	8	*80*
		<200	3	*30*	1	*10*	2	*20*	2	*20*	2	*20*
		≥200	1[a]	*10*	-	---	2	*20*	-	---	-	---
		Value[a]		*600*	-	---		*415* *1150*	-	---	-	---
McMaster	Control n=5	0	2	*40*	3	*60*	4	*80*	4	*80*	2	*40*
		<200	1	*20*	1	*20*	-	---	1	*20*	2	*40*
		≥200	2[a]	*40*	1[a]	*20*	1[a]	*20*	-	---	1[a]	*20*
		Value[a]		*350* *2050*		*1300*		*1800*				*300*
	Treatment n=10	0	8	*80*	9	*90*	8	*80*	10	*100*	0	*100*
		<200	-	---	1	*10*	-	---	-	---	-	---
		≥200	2[a]	*20*	-	---	2	*20*	-	---	-	---
		Value[a]		*300* *4000*				*300* *1500*		---	-	---

Legend: a- Mann-Whitney Test U Statistics

Table 10- Helminth faecal egg counts, over the evaluation periods for the control group and the treatment group, using the MiniFlotac and McMaster techniques

Technique	Group	Evaluation	MR	$Fr_{(4)}$	p
MiniFlotac	Control	Day 1	*8.40*	23.000[a]	0.859
	Treatment	Day 1	*7.80*		
	Control (n=5)	Day 1	3.50	2.915	0.572
		Day 30	3.20		
		Day 60	2.80		
		Day 90	2.30		
		Day 180	3.20		
	Treatment (n=10)	Day 1	3.35	3.919	0.417
		Day 30	2.65		
		Day 60	3.40		
		Day 90	2.80		
		Day 180	2.80		
McMaster	Control	Day 1	*9.80*	16.000[a]	0.189
	Treatment	Day 1	*7.10*		
	Control (n=5)	Day 1	3.90	4.786	0.310
		Day 30	3.10		
		Day 60	2.60		
		Day 90	2.40		
		Day 180	3.00		
	Treatment (n=10)	Day 1	3.25	4.000	0.406
		Day 30	3.00		
		Day 60	3.25		
		Day 90	2.75		
		Day 180	2.75		

Legend: MR- Mean Rank, a- Mann-Whitney Test U Statistics

In the treatment group, the proportion of horses with an egg count of zero was generally higher than in the control group, ranging from 80% on days 1 and 60, to 90% on day 30 and 100% on days 90 and 180. Also, on day 30 none of the horses showed clinically relevant values. Moreover, in this group, only 20% of horses on day 1 (300 and 4000 EPG) and 20% on day 60 (300 and 1500 EPG) showed counts with clinically relevant values.

In general, there were higher percentages of 0 EPG and less cases of clinically relevant values of EPG over the 5 evaluation periods in the treatment group when compared to the control group, even though these differences were not statistically significant (Fr(4)= 4.786; p= 0.310 and Fr(4)= 4.000; p= 0.406, respectively) (Table 10).

Lastly, both OPG counting methods (McMaster and MiniFlotac) were compared at the different evaluation times (table 11). On day 1, OPG counts by the McMaster technique (MR=5.00) were significantly higher than those performed by the MiniFlotac method (MR=1.50) (Wilcoxon= -1.863; p=0.039). In the remaining evaluation moments there were no significant differences in OPG counts between methods (p>0.05).

Table 11- Comparison of EPG between Miniflotac and McMaster methods in the 5 evaluation periods

Evaluation	Method	MR	MF*MM	Wilcoxon	*Unilateral* P
Day 1	MiniFlotac	1,50	2^a 5^b 8^c	-1,863	0,039
	McMaster	5,00			
Day 30	MiniFlotac	1,50	2^a 3^b 10^c	-1,214	0,156
	McMaster	4,00			
Day 60	MiniFlotac	2,50	4^a 2^b 9^c	-0,105	0,500
	McMaster	5,50			
Day 90	MiniFlotac	1,50	2^a 1^b 12^c	0,000	0,625
	McMaster	3,00			
Day 180	MiniFlotac	1,50	2^a 3^b 10^c	-1,214	0,156
	McMaster	4,00			

Legend: MR- Mean Rank , a-. MF>MM, b- MM>MF, c- MF=MM

3.7. Pergolide administration

The pergolide administration was controlled and adjusted regarding the ACTH values tested on day 60. Considering the upper reference range limit of 29 µg/ml(McGowan et al. 2013; McGowan et al. 2013), the pergolide dosage taken by every horse was increased on day 60 for the horses that still had ACTH values above the limit, by an increment of 0.001mg/kg.

From the 10 horses included in the treatment group, 5 (n= 5/10) needed an increment of 0.001mg/kg in their daily dosage of Pergolide. No horse needed more than one 0.001mg/kg increment.

Concerning the possible side effects of Pergolide administration, 2 horses from the treatment group had to interrupt the medication. One of them showed loss of appetite and

diarrhea, and another one showed excessive perspiration. In both cases, the drug was discontinued for 2-3 days and then administered at half dose for 1 week, followed by the full dose on the following week.

3.8. Owner Compliance with the Treatment

In the present study, the compliance with the pergolide treatment was 100%.

4. Discussion

The horses were included in this study based on a clinical suspicion of PPID, associated with an increase in plasma ACTH above the reference interval, which has been considered as a reliable means of diagnosis in previous studies (McFarlane et al. 2010; Christen et al. 2018). Several clinical and laboratorial outcomes were evaluated following an intervention, pergolide treatment in horses with PPID over a period of 6 months. The clinical outcomes were blood pressure, weight, body condition and cresty neck scores and the laboratorial outcome was hFEC. Furthermore, the owners' perception of the clinical signs of this disease was evaluated before and after treatment, and treatment compliance was assessed.

In the physical exam the authors also evaluated the changes in weight, body condition and cresty neck score. The body condition score showed statistically significant differences between groups, while the cresty neck score, despite showing some differences, these couldn't be considered statistically relevant. Other studies have shown no differences in both body condition and cresty neck scores between treatment and control groups (Banse et al. 2021; Miller et al. 2021). Regarding the weight changes during the period of testing, both groups showed significant weight loss. Still, even though there was a significant weight loss of the treatment group horses in the final phase of the study (day 90 and 180) compared to day 60, these horses always kept their weight above control group horses from day 60 onwards. Furthermore, their decrease in average weight from day 90 to day 180 was not as sharp as for the control group horses, suggesting better weight keeping by the horses under pergolide treatment. On the other hand, some previous studies refer a weight loss in the horses treated with pergolide, while untreated horses show weight keeping or even weight gain (Horn et al. 2019; Banse et al. 2021). Meanwhile, other authors have referred that changes in body weight were not significant between groups, showing some variability in the results obtained for this parameter (Miller et al. 2021).

As to the non-invasive measurement of blood pressure, there were no statistically significant differences between day 1 and day 180 for both systolic and diastolic values in both groups.

In fact, the small sample size of 15 PPID-affected horses and their owners led to the lack of statistical significance in some parameters. Therefore, further studies in larger

populations should be carried out. Nevertheless, although some outcomes, when compared between groups were not statistically significantly different, from a clinical point of view they had a noticeable impact on the horses' health and welfare.

As previously described, the circannual variation pattern of ACTH was proved to be remarkably similar in PPID and non-PPID horses, suggesting that the regulation of the photoperiod by the pituitary gland appears to be preserved when horses have this disease (Copas and Durham 2012; Humphreys et al. 2022). Also, circannual reference values for ACTH have been published and, since the greatest difference in ACTH levels between healthy horses and the ones with PPID occurs between August and October, it is suggested that, applying the circannual reference intervals, this period might be the most appropriate to test PPID (Copas and Durham 2012; Durham et al. 2020).

The present study was conducted between December and July, where ACTH levels differ less between both groups, which may explain why both groups had decreased values in similar proportions. Also, the horses included in the study were selected based on suspicion of PPID (phenotypical presentation and clinical signs), combined with an increase in plasma ACTH, which was shown to be a reliable form of diagnosis in a previous study (van der Kolk et al. 1995). Further confirmatory testing, such as TRH stimulation test or ODST, may have been desirable (Kirkwood et al. 2022), but due to restricted financial resources of the study and limited time both tests were unable to be performed.

Nevertheless, even though there was a decrease in ACTH values along the study period in both groups, in the treatment group 8 in 10 horses (n= 8/10) maintained the ACTH value within the normal range (<29 µg/ml). On the other hand, in the control group, despite the decrease along the study period, 3 in 5 horses (n=3/5) still had values above 29 µg/ml at the end of the study. Furthermore, ACTH values (mean ± standard deviation) in the control group were higher than in the treatment group.

The existing studies concerning PPID and hFEC compared horses with the disease and healthy controls (McFarlane et al. 2010; Christen et al. 2018). In this study we compared horses with PPID with (treatment group) or without (control group) pergolide treatment.

Regarding hFEC, using both McMaster and Mini-Flotac techniques, it was concluded that there were no significant differences in baseline EPG between groups. On day 180, the proposed hypothesis that horses with PPID, treated with pergolide, had less EPG and time to reappearance due to improved immune function could not be statistically confirmed. Nevertheless, with the McMaster technique, in general, there were higher percentages of 0 EPG and fewer cases of clinically relevant values of EPG over the 5 evaluation periods in the treatment group when compared to the control group. Also, the horses from the treatment group maintained hFEC <200 eggs/gram through the study and the control group had 1 animal that kept hFEC almost always above acceptable values. Even though the differences between groups were not statistically significant, possibly due to the small size population

involved in the present study, pergolide treatment seemed to have some indirect influence in parasite control.

These results are in agreement with a previous study (Christen et al. 2018) that intended to evaluate the use of EPG as a marker for immune function and reported no statistically significant differences in EPG in horses with pre-clinical PPID, treated with pergolide or with a placebo. Despite secondary infections being common in horses with PPID, the exact mechanism of immunosuppression is still undisclosed. It is likely that the high circulating concentrations of ACTH, α-MSH, and cortisol suppress the immune response. However, these hormones typically shift immunity toward a Th2 response, which is not consistent with an increase in susceptibility to parasites. Additional studies are necessary to understand the mechanism responsible for the increased susceptibility to parasites in horses with PPID(McFarlane et al. 2010).

From the questionnaire analysis the most relevant information to highlight is the fact that the owners were unaware of some of the clinical signs of PPID, as they didn't notice them initially. In fact, in the final questionnaire they mentioned some improvements in clinical signs which they had not acknowledged to exist in the first questionnaire. For example, when asked in the first questionnaire if the long haircoat shed during summer, in the treatment group 5 owners (n= 5/10) answered yes and the other 5 (n= 5/10) said no. Meanwhile, six months later, when asked the same question, 8 owners (n= 8/10) stated that there was an improvement regarding this matter. Similarly, when asked if the horse appeared to be occasionally lame, only one owner (n= 1/5) said yes. Nonetheless, after treatment 2 owners stated that there was an improvement. Likewise, regarding the loss of muscle, 3 owners reported it (n= 3/10) in the first questionnaire, while when asked again, 6 months later 6 owners (n= 6/10) reported improvement in their horses' muscle mass.

This demonstrates the unawareness about the disease and its clinical signs, as well as the effects of pergolide, as owners noticed several improvements in many physical aspects of their horse, regarding factors which they had not detected at the beginning. We believe that these results are of utmost importance, as they point out a gap in the clinical diagnosis and management of PPID, emphasizing the need to change our approach in consultation.

Moreover, the fact that, in the present study, treatment compliance was 100% suggests the relevance of taking time from the first consultation to assure a bilateral understanding of the disease, of its management, treatment, risk factors and possible consequences. Moreover, the monthly in-person visits for the first six months in this type of chronic disease therapy seemed to further contribute to compliance.

In fact, we believe that this approach is a step forward to a shift from compliance to adherence, where the owner is better informed in order to increase trust, leading to active choices on the owner's behalf and to 0% dropout rate (F. Fregni and Illigens 2018).

5. Conclusions

The present study contributes to a more significant insight on the difficulties associated with owner awareness of PPID clinical signs, owner compliance with treatment and the relevance of parasite control. This may allow us to better help horses and their owners, who deal daily with this disease, with repercussions on well-being, body condition and exercise performance of ageing horses, implying also a financial loss. The questionnaires were a vital part of this process, as they created owner awareness of the clinical signs of PPID, which were recurrently attributed to old age alone.

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