Aus der Klinik für Pferde
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Effects of Endocrinopathies on Plasma Amino Acid Profile of Horses

Inaugural-Dissertation
zur Erlangung des Grades eines
Doctor of Philosophy (PhD)
in Biomedical Sciences
an der
Freien Universität Berlin

vorgelegt von
Dr. Sabita Diana Stöckle
Tierärztin aus Frankfurt am Main

Berlin 2023
Journal-Nr.: 4400

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List of abbreviations

1mHis: 1-methylhistidine

ACTH: Adrenocorticotropic hormone

Ala: Alanine, Arg: Arginine

Asn: Aspargine

Asp: Aspartic Acid

CGIT: Combined Glucose Insulin Test

Citr: Citrulline

Cys: Cysteine

EDTA: Ethylenediaminetetraacetic acid

EMS: Equine Metabolic Syndrome

GABA: Gamma-aminobutyric acid

Gln: Glutamine

Glu: Glutamic acid

Gly: Glycine

His: Histidine

IGF-1R: Insulin-like growth factor 1 receptor

Ile: Isoleucine

Leu: Leucine

Lys: Lysine

Max: Maximum

Med: Median

Met: Methionine

Min: Minimum

nPPID: non-PPID horse

Orn: Ornithine

Phe: Phenylalanine

PPID: horse suffering from PPID

PPID: Pituitary Pars Intermedia Dysfunction

PPIDarr: horse treated for PPID and ACTH ≥ 100 pg/ml

PPIDrr: horse treated for PPID and ACTH ≤ 30 p g/ml

Pro: Proline

SD: Standard deviation

Ser: Serine

Tau: Taurine

Thr: Threonine

Trp: Tryptophan

Tyr: Tyrosine

Val: Valine

1. Introduction

The development of laminitis in horses is often associated with an endocrine disease (Equine Metabolic Syndrome = EMS; Pituitary Pars Intermedia Dysfuction = PPID = Equine Cushing's Syndrome), in which insulin-dysregulation occurs (EMS) or can occur (PPID) (Karikoski et al. 2011; Karikoski et al. 2015; Karikoski et al. 2016). More rarely, there is sepsis and/or endotoxaemia-associated laminitis or stress-inducible laminitis, for example on the contralateral limb after fracture treatment (Patterson-Kane et al. 2018; Leise and Fugler 2021).
A suspected pathomechanism of endocrinopathic laminitis in EMS and PPID is hyperinsulinaemia, but the mechanism by which hyperinsulinaemia can cause laminitis remains unclear (Stokes et al. 2021).
Based on the results of previous studies, insulin is assumed to exert an effect on the laminar tissue of the hoof via the insulin-like growth factor 1 receptor (IGF-1R) (De Laat et al. 2013; Lane et al. 2017; Sandow et al. 2019) and also that insulin itself may directly induce laminitis through a yet unidentified mechanisms (Stokes et al. 2021).
In addition to its roles in glucose metabolism, insulin is an important regulator of protein metabolism. Through increased amino acid utilisation, hyperinsulinemia can contribute to increased amino acid and protein turnover, for example in skin or skeletal muscle (Fukagawa et al. 1986; Hillier et al. 1998; Timmerman et al. 2010; Urschel et al. 2014; Tuvdendorj et al. 2015). In humans and dogs suffering from the clinical syndrome of necrotic migratory erythema and/or superficial necrolytic dermatitis, a reduced concentration of amino acids in the blood was observed in some cases. After supplementation with amino acids, the clinical signs improved or disappeared (Outerbridge et al. 2002; March et al. 2004; Flores et al. 2016; Loftus et al. 2017)
As hoof horn is an appendix of the epidermis, the changes present in laminitis might also be influenced by reduced amino acid concentrations. A recent study on healthy horses showed that during an euglycaemic, hyperinsulinaemic clamp for 48 h or a glucose infusion for 66 h, there is a reduction in plasma amino acids and clinical signs of laminitis may develop (Stokes et al. 2021).
An altered plasma amino acid profile might be used to diagnose EMS and PPID on the one hand and to identify animals with a particularly high risk of laminitis on the other. In addition, if an amino acid deficiency is present, supplementation with the corresponding amino acids could contribute to an improvement of the laminitis symptoms and thus to an improvement of the long-term prognosis.

2. Literature review

Changes of the metabolome in endocrine diseases

Cushing's syndrome in humans, pituitary adenoma and pituitary pars intermedia dysfuction

Pituitary pars intermedia dysfunction (PPID) is also known as equine Cushing's syndrome. In this disease, the horse's cortisol concentration can remain in the reference range (Boujon et al. 1993), be elevated (Dybdal et al. 1994), or decreased (Beech and Garcia 1985; Eiler et al. 1997). However, even though the total cortisol concentration is reported to be similar to healthy horses, the free cortisol fraction in the blood was shown to be significantly higher in PPID patients (Hart et al. 2016). Endogenous hypercortisolism occurs in human Cushing's syndrome. The hypercortisolism mostly originates in the adrenal cortex and is associated with metabolic and cardiovascular complications, as well as increased mortality (Debono et al. 2014; Di Dalmazi et al. 2014; Morelli et al. 2014; Ferraù and Korbonits 2015; Clayton et al. 2016). Human patients with hypercortisolism had lower concentrations of short- and medium-chain acylcarnitines and branched-chain and aromatic amino acids, but higher plasma polyamine concentrations than healthy controls (Di Dalmazi et al. 2017). Alterations in the metabolome, especially in the plasma amino acid concentrations, were also reported in humans suffering from pituitary adenomas (Pînzariu et al. 2019). In one study, 12 metabolites were initially significantly altered in the blood of humans with pituitary adenoma compared to the control group, but after performing a Bonferroni correction, only 3 remained: pyridoxate, deoxycholic acid and 3-methyladipate (Oklu et al. 2014). In the metabolic pathway analysis, several changes were detected, including changes in the amino acid metabolism. Significantly affected were the alanine, aspartate and glutamate metabolism, the starch and sucrose metabolism as well as the amino sugar and the nucleotide sugar metabolism, the lysine biosynthesis, the vitamin B6 metabolism, the aminoacyl-tRNA biosynthesis, the glycolysis or gluconeogenesis and the purine metabolism (Oklu et al. 2014). Looking at the pituitary tissue, significant metabolic differences between neoplastic (ACTH-secreting pituitary adenoma) and healthy pituitary tissue were detected. In particular, proteins and metabolites involved in the glycolysis/gluconeogenesis, the pyruvate metabolism, the citrate cycle and the fatty acid metabolic pathway were significantly accumulated in pituitary adenoma (Feng et al. 2018). Furthermore, other diseases such as Parkinson's disease, which, like PPID, is associated with oxidative damage to the dopaminergic neuronal pathways (McFarlane 2007) arginine, alanine and phenylalanine concentrations were significantly lower in patients with advanced disease and dyskinesia than in early-stage patients (Figura et al. 2018).

Initial studies on the amino acid profile of horses suffering from PPID were already performed: As part of a master's thesis, the plasma amino acid profile of eight PPID patients and four healthy horses was compared. This study showed that the majority of amino acid

concentrations were not significantly different between healthy horses and individuals suffering from PPID. However, the concentrations of aspartic acid and ornithine were significantly (aspartic acid) and tendentially (ornithine) higher in PPID patients than in healthy horses. In addition, the plasma concentrations of glutamine, methionine, 3-methylhistidine and threonine were significantly (glutamine, methionine, 3-methylhistidine) or tended (threonine) to be lower than in healthy horses (De Vries 2015).

Metabolic syndrome

In human medicine, the presence of multiple obesity-associated risk factors that may predict the development of cardiovascular disease is referred to as human metabolic syndrome (Day 2007). While there is general consensus on the main components of glucose intolerance, obesity, elevated blood pressure and dyslipidaemia (elevated triglycerides, low levels of high-density lipoprotein cholesterol), cut-off values and mandatory inclusion criteria are not defined consistently (Day 2007). Equine metabolic syndrome (EMS), which describes the presence of multiple risk factors for the development of endocrinopathic laminitis (Durham et al. 2019) and whose central feature is an existing insulin-dysregulation (Frank and Tadros 2014), is a comparable disease to human metabolic syndrome. (Regional) Obesity (Treiber et al. 2006; Carter et al. 2009), rapid weight gain, and difficulty in losing weight (Argo et al. 2012) are also considered to be components of EMS, but this syndrome is occasionally also described in slender horses (Bailey et al. 2008). Other inconsistent features include cardiovascular disease, elevated blood pressure, increased heart rate and altered cardiac dimensions (Bailey et al. 2008; Heliczer et al. 2017). A dysregulation of lipid metabolism, which manifests as hypoadiponectinaemia and hyperleptinaemia, was also described (Carter et al. 2009; Menzies-Gow et al. 2017). In obese humans, the concentrations of amino acids, especially the branched-chain amino acids, are increased in the plasma (Felig et al. 1970; Leclercq and Sève 1994; Solini et al. 1997). Peripheral insulin resistance is also present in human type 2 diabetes mellitus (Weyer et al. 1999), which is associated with obesity (Kahn et al. 2006). In affected individuals, higher serum concentrations of isoleucine, valine, tyrosine, alanine and methionine were reported (Chen et al. 2022). Further studies identified increased concentrations of isoleucine, leucine, valine, phenylalanine and tyrosine in patients considered at risk of developing type 2 diabetes mellitus, and lower concentrations of glycine and glutamate (Wang et al. 2011; Palmer et al. 2015; Guasch-Ferré et al. 2016). However, another study reported increased concentrations of alanine, proline, valine, leucine/isoleucine, phenylalanine, tyrosine, glutamate/glutamine and ornithine, and branched-chain and related amino acids (Tai et al. 2010). Also, a study involving normoglycaemic women identified higher serum concentrations of branched-chain amino acids with a high Homeostatic Model Assessment for Insulin Resistance (Wiklund et al. 2016).

Furthermore, significant differences in the plasma amino acid concentrations have been reported in patients with coronary heart disease and type 2 diabetes mellitus: Compared with control patients, patients with coronary heart disease type 2 diabetes mellitus had higher concentrations of the branched-chain amino acids isoleucine, leucine and valine and the aromatic amino acid phenylalanine. Additionally, these patients showed higher plasma concentrations of leucine, valine, fumarate, tyrosine and phenylalanine when compared to patients with coronary heart disease without type 2 diabetes mellitus. Furthermore, the biosynthesis and degradation of phenylalanine, tyrosine and tryptophan biosynthesis, valine, leucine and isoleucine was modified (Zhang et al. 2021).

Changes of the metabolome were also described in insulin-dysregulated horses. One study found significantly lower methionine and trans-4-hydroxyproline concentrations in insulin-dysregulated than in insulin-sensitive horses (Kenéz et al. 2018). Other studies also showed significant differences of the metabolome in insulin-dysregulated and non-insulin-dysregulated animals, these mainly concerned the lipid and amino acid metabolism (Geidenstam et al. 2014; Jacob et al. 2018). Delarocque and colleagues described lower plasma concentrations of arginine and carnitine in horses with a high insulin response to the oral glucose test (Delarocque et al. 2021). Stokes et al. obtained significant changes in the amino acid profile in healthy horses by experimentally inducing hyperinsulinaemia with an euglycaemic hyperinsulinaemic clamp and via a glucose infusion. Here, there was a significant decrease of 15 (glucose infusion) and 19 (euglycaemic hyperinsulinaemic clamp) of the 20 amino acids determined in plasma, respectively (Stokes et al. 2021). Currently no data are available on horses suffering from endocrinopathic laminitis. However, horses with gastrointestinal disorders that developed laminitis were found to have lower plasma concentrations of citrulline than healthy horses (Jackson 2013).

Summary of the literature and aims of the study

- A change in the metabolome has been reported in various human diseases including endocrine diseases.
- Changes in the metabolome in the context of insulin-dysregulation have also been reported in horses.
- The plasma amino acid profile of horses changes during experimentally-induced hyperinsulinaemia.
- Changes in the plasma amino acid profile are also present in Parkinson's disease, which is associated with oxidative nerve damage similar to PPID.

This leads to the following questions:

- Are there differences in the amino acid profile between healthy horses and horses suffering from PPID?
- Are there differences in the amino acid profile between obese and insulin-dysregulated horses with and without laminitis?

3. Paper I - Plasma Amino Acids in Horses Suffering from Pituitary Pars Intermedia Dysfunction

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Plasma Amino Acids in Horses Suffering from Pituitary Pars Intermedia Dysfunction

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Simple Summary: Pituitary pars intermedia dysfunction (PPID), also known as equine's cushing syndrome, is one of the most common diseases of aged horses and ponies. The pathogenesis of PPID includes oxidative damage to dopaminergic pathways, similar to Parkinson's disease in humans. Here, alterations in the concentrations of the serum amino acids were reported previously. To examine changes in the plasma amino acid profile in horses with PPID, EDTA plasma of horses that were presented for various reasons that required laboratory examinations of blood anticoagulated with EDTA was collected. With this plasma, the basal ACTH concentration, as well as the amino acid profile, was determined. The basal ACTH concentration is commonly used to diagnose PPID. Horses were considered PPID patients if the ACTH concentration was $\geq$ 100 pg/mL, i.e., they would be considered affected at any time. Horses were defined as non-PPID (nPPID) patients if the ACTH concentration was below 30 pg/mL. PPID is commonly treated with pergolide. Horses receiving pergolide with ACTH $\leq$ 30 pg/mL were allocated to the group PPIDrr (PPID, ACTH in reference range) and horses receiving pergolide with ACTH $\geq$ 100 pg/mL to the group PPIDarr (PPID, ACTH above reference range). In total, 93 horses were examined, including 88 horses at the clinic and 5 horses at a private practice. Of these, 53 horses fulfilled the inclusion criteria (ACTH $\leq$ 30 pg/mL or ACTH $\geq$ 100 pg/mL). A total of 25 horses were diagnosed as nPPID, 20 as PPID, 5 as PPIDrr, and 3 as PPIDarr. Arginine was significantly higher in PPIDrr than in PPID and nPPID, asparagine was significantly higher in PPID, PPIDrr, and PPIDarr than in nPPID, citrulline was significantly higher in PPIDrr than in nPPID and PPID, cysteine was significantly lower in PPIDrr than in PPID, nPPID, and PPIDarr, and glutamine was significantly higher in PPID and PPIDarr than in nPPID. Especially, asparagine, citrulline, and glutamine may be potential diagnostic markers and may offer interesting approaches for research regarding amino supplementation in PPID.

Abstract: Pituitary pars intermedia dysfunction is one of the most common diseases of aged horses and ponies. In Parkinson's disease, which is, similar to PPID, a disease that involves oxidative damage to dopaminergic pathways but with different clinical signs, alterations to the serum amino acid profile have been reported. To examine changes in the plasma amino acid profile in horses with PPID, EDTA plasma of horses that were presented for various reasons that required laboratory examinations of blood anticoagulated with EDTA was collected. With this plasma, the basal ACTH concentration as well as the amino acid profile was determined. Horses were considered PPID patients if the ACTH concentration was $\geq$ 100 pg/mL, i.e., they would be considered affected at any time. Horses were defined as non-PPID (nPPID) patients if the ACTH concentration was below 30 pg/mL. Horses receiving pergolide with ACTH $\leq$ 30 pg/mL were allocated to the group PPIDrr (PPID, ACTH in reference range) and horses receiving pergolide with ACTH $\geq$ 100 pg/mL to the group PPIDarr (PPID, ACTH above reference range). In total, 93 horses were examined, including 88 horses at the clinic and 5 horses at a private practice. Of these, 53 horses fulfilled the inclusion criteria (ACTH $\leq$ 30 pg/mL or ACTH $\geq$ 100 pg/mL). A total of 25 horses were diagnosed as nPPID, 20 as PPID, 5 as PPIDrr, and 3 as PPIDarr. Arginine was significantly higher in PPIDrr than in PPID and nPPID, asparagine was significantly higher in PPID, PPIDrr, and PPIDarr than in nPPID, citrulline was

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significantly higher in PPIDrr than in nPPID and PPID, cysteine was significantly lower in PPIDrr than in PPID, nPPID, and PPIDarr, and glutamine was significantly higher in PPID and PPIDarr than in nPPID. Especially, asparagine, citrulline, and glutamine may be potential diagnostic markers and may offer interesting approaches for research regarding amino supplementation in PPID.

Keywords: plasma amino acids; PPID; endocrine disease; arginine; asparagine; citrulline; cysteine; glutamine

1. Introduction

Pituitary pars intermedia dysfunction is one of the most common diseases of aged horses and ponies (≥15 years) [1–3]. Laminitis occurs in 30–40% of PPID patients and may necessitate euthanasia [4–6]. In PPID, hypertrophy, hyperplasia, and microadenoma or macroadenoma formation of the pars intermedia of the pituitary occurs [7]. This leads to an increased secretion of the pars intermedia-derived POMC (Pro-Opiomelanocortins) into the circulation [7]. From the healthy equine pars intermedia, only a small amount of the adrenocorticotropic hormone (ACTH) is released and it is further cleaved into α-melanocyte stimulating hormone (α-MSH), β-endorphine (β-END), and corticotropine-like intermediate lobe peptide (CLIP) [7]. In the diseased equid, the pars intermedia secretes an increased amount of POMC-derivates into the systemic circulation. An increase up to 40-fold was reported previously [8]. Nowadays, the basal ACTH concentration is a commonly used test to diagnose PPID with a sensitivity of approximately 70–80% and a spe-cificity of approximately 80–90% [5,9,10]. ACTH stimulates the adrenal gland to synthesize and release cortisol into circulation [11]. Previous research has suggested that the increased endogenous glucocorticoid concentrations may be responsible for laminitis, since these are known to be associated with systemic insulin resistance [12–17]. The suspected mechanism is that binding of insulin to the insulin-like growth factor 1 receptor (IGF-1R) has an effect in the lamellar tissue of the hoof [18–20] and also that insulin can directly trigger laminitis even through mechanisms that have not yet been identified [21]. In addition to its effects on the glucose metabolism, insulin is an important regulator of protein metabolism. Due to an increased utilization of amino acids, hyperinsulinemia can contribute to an increased amino acid and protein turnover, for example, in the skin or skeletal muscles [22–26].

Furthermore, in human Parkinson's disease, which is, similarly to PPID, a disease that involves oxidative damage to dopaminergic pathways but has a different clinical presentation [27], the arginine, alanine, and phenylalanine concentrations were significantly lower in patients with advanced disease and dyskinesia than in patients suffering from early disease and could therefore serve as a biochemical marker of disease progression [28]. As possible reasons for the significant differences in the serum amino acid profile malabsorption and changes in amino acid metabolism, the effects of mitochondrial dysfunction and oxidative stress, reflection of progressive neurodegenerative processes in the brain, and the effect of dopaminergic medications and aromatic L-amino decarboxylase inhibitors were suggested [28].

Beside Parkinson's disease, a change in the amino acid level in plasma or serum has been demonstrated in other human diseases, including autism and cancer. A study by Naushad et al. reported increased concentrations of glutamic acid and asparagine, and lower concentrations of phenylalanine, tryptophan, methionine, and histidine in autistic children [29]. Another study reported significantly higher concentrations of histidine, 1-methyl-histidine and 3-methyl-histidine, and significantly lower concentrations of homocysteine, carnosine, methionine, cystathionine, cystine, tyrosine, and threonine in autistic children compared to healthy children [30]. Therefore, it appears that increased concentrations of excitatory amino acids (glutamate and asparagine) and decreased concentrations of essential amino acids (phenylalanine, tryptophan, and methionine), as well as decreased concentrations of neurotransmitter precursors (tyrosine and tryptophan), may be distinc-

tive features of the plasma amino acid profile of autistic children and that this could offer an opportunity for early diagnosis [29].

Even in disease caused by the novel coronavirus (SARS-CoV-2), significant differences in the amino acid profile in the plasma in hospitalized adults and children with multisystem inflammatory syndrome, in particular a reduced arginine concentration and arginine bioavailability, are considered important. It was suggested that arginine deficiency may contribute to endothelial dysfunction, T cell dysregulation, and coagulopathy [31]. Changes in plasma amino acid profile were also found in cancer patients. This could be due to changes due to cancer-induced protein metabolism in tumors, skeletal muscle, and liver in cancer patients. Cancer-related plasma amino acid profiles are particularly present in cancers which affect the digestive organs. These are not only influenced by the type of cancer, but also by the cancer stage [32–37]. The plasma amino acid profile seems to undergo specific changes during different diseases, not only in humans, but also in animals, which was previously shown in horses suffering from equine metabolic syndrome [38,39] or in experimentally-induced hyperinsulinemia [21].

Therefore, we hypothesized that there may be differences in the amino acid profile of healthy horses and horses affected with PPID. Furthermore, we assumed that treatment with pergolide may affect the amino acid profile as well and that these may be used as a potential diagnostic biochemical marker.

2. Material and Methods

2.1. Study Population

Included in the study were horses that were presented for various reasons at the Equine Clinic of Freie Universität Berlin (FU Berlin) that required laboratory examinations of blood anticoagulated with EDTA. The horses had to be clinically healthy except for possible orthopaedic or ophtalmological reasons for examination and/or surgery. After a physical examination, the horses were stabled. The time between transport and sample collection varied; however, all horses were given time to accommodate to their surroundings before sample collection (at least 30 min after transport). At the moment of sample collection, all horses showed no signs of pain. Their vital parameters were within normal limits and the horses were relaxed and comfortable in their surroundings. Besides the ACTH concentration, age, weight, breed, gender, and feeding regimen were recorded. Further, 5 horses that presented to a private practice to evaluate the response to therapy by determining the ACTH concentration were included in the study. Individuals with suspected or diagnosed endocrinopathies (previous dynamic testing for equine metabolic syndrome, suspicious fat accumulations) other than PPID were excluded from the statistical analysis. Since the samples were collected at different times of the year, horses were classified as healthy if the measured ACTH concentration was normal (ACTH $\leq$ 30 pg/mL) at any time of the year. The reference value of LABOklin (Holding—GmbH, Bad Kissingen/Germany) were used. Horses were considered PPID patients if an ACTH concentration $\geq$ 100 pg/mL was present, i.e., they would be considered affected at any time. Horses were defined as non-PPID (nPPID) patients if the ACTH concentration was below 30 pg/mL and as PPID patients (PPID) if the ACTH concentration above 100 pg/mL. Horses receiving pergolide with ACTH $\leq$ 30 pg/mL were allocated to the group PPIDrr (PPID, ACTH in reference range) and horses receiving pergolide with ACTH $\geq$ 100 pg/mL to the group PPIDarr (PPID, ACTH above reference range).

2.2. Laboratory Diagnostics

After required laboratory diagnostics were performed (within 10 min of collection), the blood samples, uncoagulated with EDTA, were centrifuged and the plasma was separated from the solid blood components.

2.2.1. ACTH

The plasma for the ACTH determination was sent cooled to the laboratory within a maximum of 12 h after collection. ACTH was determined with a chemoluminiscence-assay by Laboklin (LABOklin Holding—GmbH Bad Kissingen/Germany).

2.2.2. Amino Acid Concentrations

The amino acid profiles were determined by MembraPure GmbH (Hennigsdorf/Germany). In order to determine the total cysteine concentration, bound cysteine had to be reduced to free cysteine. For this, 500 µL plasma and 100 µL dithiothreitol solution (4%) were mixed in an Eppendorf tube and incubated at 40 °C for 30 min. Then, 150 µL sulfosalicylic acid solution (10%) was added and the sample was stored for 30 min at 5–8 °C. Afterwards, 500 µL sample dilution buffer (with internal standard norleucine 100 nmol/mL) was added. The sample was centrifuged at 13,150× *g* for 5 min. After these steps, the amino acid analyses were performed with the Aracus amino acid analyzer, using the amino acid analysis method based on ion exchange chromatography with post column derivatization with Ninhydrin. The amino acid concentrations were determined by comparing the sample with a standard solution with predefined concentrations using the Clarity Chromatography Software (DataApex Company, Prague, Czech Republic).

2.3. Statistical Analysis

Data were analyzed using IBM SPSS Statistics version 27 (IBM Corp., Armonk, New York, USA). Data were tested for normal distribution with the Shapiro Wilk Test. The Chi-Squared-test was used to compare gender distribution between groups. Further statistical tests used were the ANOVA for normally distributed data and the Kruskal–Wallis test for non-normally distributed data. For post hoc testing, the Tukey test and the Games–Howell test or Bonferroni test were used. Laboratory data that could not be measured as they exceeded the maximum which is validated for the test or fell below the detection limit, were regarded as either the maximum (ACTH > 1250 pg/mL, n = 1) or the minimum (Asp < 5 nmol/mL, n = 20, nPPID = 7, PPID = 12, PPIDarr = 1). As per usual, the significance level was set at 0.05.

2.4. Ethical Statement

The study was not declared according to the German Animal Welfare law §8.1, since all samples were taken as a part of a routine clinical examination. Written owner's consent to involve their horses in the study was obtained during the admission process at the clinic as well as at the private practice.

3. Results

3.1. Study Population

In total, 93 horses were examined, including 88 horses at the clinic and 5 horses at the private practice. Of these, 53 horses fulfilled the inclusion criteria (ACTH $\leq$ 30 pg/mL or ACTH $\geq$ 100 pg/mL):

A total of 25 horses were diagnosed as nPPID, 20 as PPID, 5 as PPIDrr, and 3 as PPIDarr.

There was no significant difference regarding gender between these groups (p = 0.428, Welch Test). Information on breed was unavailable for two horses. Ten of the twenty individuals in the PPID group were ponies, compared to 7/25 horses in the nPPID, 1/5 in the PPIDrr, and 2/3 in the PPIDarr group. Six horses had a history of laminitis (3 nPPID, 2 PPID, and 1 PPIDarr), and there was no significant difference between the groups (p = 0.553, Kruskal–Wallis Test). However, significant differences between the groups were detected for feeding, age, and ACTH concentration. All horses identified as PPIDrr were exclusively maintained on a hay diet, whereas the nPPID and PPID patients also received concentrates (1 nPPID), Mash (10 nPPID, and 3 PPID) or grass (1nPPID and 12 PPID) in addition to hay (p = 0.005, Kruskal–Wallis Test). One horse (nPPID) received grass only. Information on

age was unavailable for one horse (PPIDrr). Horses suffering from PPID were significantly older than nPPID horses ($p < 0.001$, ANOVA with Games-Howell test). Between the other groups, there were no significant differences regarding age. There were no significant differences in the ACTH concentration between nPPID and PPIDrr ($p = 0.972$, ANOVA with Games-Howell test). The ACTH concentration of PPID patients was significantly higher than in nPPID ($p < 0.001$, ANOVA with Games-Howell test), PPIDrr ($p < 0.001$, ANOVA with Games-Howell test), and PPIDarr ($p = 0.014$, ANOVA with Games-Howell test). Furthermore, PPIDarr horses had significantly higher ACTH concentrations than PPIDrr horses ($p = 0.017$, ANOVA with Games-Howell test). Mean and standard deviations of age and ACTH are displayed in Table 1.

Table 1. Age and ACTH concentrations of the tested horses (mean ± standard deviation).

Parameter	nPPID	PPID	PPIDrr	PPIDarr
ACTH (pg/mL)	19.76 ± 6.96	382.85 ± 352.69	18.1 ± 7.88	154.0 ± 23.07
Age (years)	15.91 ± 6.96	27.79 ± 6.7	21.25 ± 3.77	27.33 ± 4.16

nPPID: non-PPID horse, PPID: horse suffering from PPID, PPIDrr: horse treated for PPID and ACTH ≤ 30 pg/mL, and PPIDarr: horse treated for PPID and ACTH ≥ 100 pg/mL.

3.2. Amino Acid Analysis

The mean ± standard deviation and the median, minimum and maximum of the measured amino acid concentrations are displayed in Tables 2 and 3. The *p*-values of ANOVA or Kruskal–Wallis tests are included in these tables as well.

Significant group differences were detected for arginine, asparagine, citrulline, cysteine, glutamine, and threonine.

Table 2. Mean and standard deviations of the normally distributed amino acid concentrations (nmol/mL); *p*-values (ANOVA).

	nPPID		PPID		PPIDrr		PPIDarr		*p*
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	
1mHis	17.1	6.0	15.8	6.0	18.5	9.2	23.7	6.2	0.231
Ala	205.8	69.0	215.0	85.1	164.2	41.7	213	70.3	0.595
Arg	67.3	29.7	86.7	20.0	95.7	27.1	116.0	16.0	0.004
Asn	35.6	20.3	68.0	29.1	33.0	12.2	78.9	53.1	0.018
Cit	56.4	20.2	53.3	19.1	83.9	12.0	68.1	17.4	0.016
Cys	135.6	81.9	169.8	32.9	18.7	7.4	146.4	115.0	<0.001
GABA	20.4	8.0	19.3	8.0	12.9	1.1	21.9	11.1	0.258
Gln	239.8	56.1	334.8	43.9	286.4	46.0	337.4	42.363	<0.001
Glu	40.9	24.4	35.7	20.3	18.3	5.3	41.4	23.2	0.21
Gly	410.2	161.6	431.9	119.5	400.8	146.7	384.4	90.2	0.92
His	71.2	13.4	81.3	17.2	77.5	8.0	86.4	5.1	0.89
Ile	60.6	16.0	70.2	19.4	53.2	15.4	72.9	11.5	0.113
Leu	100.9	30.6	112.6	36.8	97.8	24.7	123.5	20.7	0.458
Orn	59.5	18.1	57.4	14.3	64.0	5.5	55.7	5.2	0.83
Phe	54.9	11.4	53.5	11.2	60.1	7.2	59.0	3.6	0.603
Ser	212.4	64.6	244.2	68.1	220.9	34.0	227.1	20.5	0.419

Table 2. *Cont.*

	nPPID		PPID		PPIDrr		PPIDarr		*p*
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	
Tau	41.3	12.6	47.3	14.6	40.1	13.1	42.9	13.3	0.473
Trp	50.9	12.8	52.2	16.3	56.0	11.5	72.6	12.5	0.102
Val	157.4	52.0	162.7	49.2	172.7	26.5	174.8	42.9	0.88

nPPID: non-PPID horse, PPID: horse suffering from PPID, PPIDrr: horse treated for PPID and ACTH $\leq$ 30 *p* g/mL, PPIDarr: horse treated for PPID and ACTH $\geq$ 100 pg/mL, SD: Standard deviation, 1mHis: 1-methyl histidine, Ala: Alanine, Arg: Arginine, Asn: Aspargine; Citr: Citrulline, Cys: Cysteine, GABA: Gamma-aminobutyric acid, Gln: Glutamine, Glu: Glutamic acid, Gly: Glycine, His: Histidine, Ile: Isoleucine, Leu: Leucine, Met: Methionine, Orn: Ornithine, Phe: Phenylalanine, Ser: Serine, Tau: Taurine, Trp: Tryptophan, Val: Valine.

Table 3. Median, minimum, and maximum of the non—normally distributed amino acid concentrations (nmol/mL); Kruskal—Wallis Test.

	nPPID			PPID			PPIDrr			PPIDarr			*p*
	Med	**Min**	**Max**	**Med**	**Min**	**Max**	**Med**	**Min**	**Max**	**Med**	**Min**	**Max**	
Asp	8.1	5	16.5	5	5	20.7	8.1	5.7	9.9	8.3	5.0	16.5	0.304
Lys	76.1	45.5	153.8	92.6	29.3	143.5	87.8	46.7	122.9	114.6	80.0	126.2	0.498
Pro	76.3	42.8	308.7	74.0	35.8	147.1	68.2	46.6	104.1	102.2	78.2	112.7	0.315
Thr	94.7	41.1	203.9	129.6	65.9	208.4	113.2	39.6	138.6	151.5	125.7	156.6	0.047
Tyr	52.1	36.5	97.6	58.0	36.4	93.5	63.8	46.5	96.7	63.2	50.8	64.4	0.528

nPPID: non-PPID horse, PPID: horse suffering from PPID, PPIDrr: horse treated for PPID and ACTH $\leq$ 30 *p* g/mL, PPIDarr: horse treated for PPID and ACTH $\geq$ 100 pg/mL, Med: median, Min: minimum, Max: maximum, Asp: aspartic Acid, Lys: lysine, Pro: proline, Thr: threonine, Tyr: tyrosine.

Arginine in PPIDarr was significantly higher than in nPPID ($p = 0.016$, Tukey test). Asparagine was significantly higher in PPID when compared to nPPID ($p < 0.001$, Games-Howell test) and PPIDrr ($p = 0.043$, Games-Howell Test). Furthermore, the asparagine concentration was significantly higher in PPIDarr when compared to nPPID (both: $p = 0.039$, Games-Howell test). Citrulline was significantly higher in PPIDrr when compared to nPPID ($p = 0.024$, Tukey test) and PPID ($p = 0.012$, Tukey test). The cysteine concentration in PPIDrr was significantly lower than in all other groups (nPPID vs PPIDrr $p = 0.003$, Games-Howell test, PPID vs PPIDrr $p = 0.043$, Games-Howell test, PPIDrr vs PPIDarr $p = 0.044$, Games-Howell test). When compared to nPPID, the glutamine concentration in PPID ($p < 0.001$, Tukey test) and PPIDarr ($p = 0.014$, Tukey test) was significantly higher. For threonine, no significant group differences were identified by post hoc testing.

4. Discussion

The main limitation of this study is the insufficient number of PPIDrr and PPIDarr that were included in the analysis. However, since an adequate number of horses was included PPID and nPPID, the comparisons between these groups should provide valid results. Furthermore, even if only a few horses medicated with pergolide were available for the study, the detected differences clearly show that the amino acid profile is potentially affected by this medication. For PPID, epidemiological differences regarding gender or breed were not reported previously; however, increasing age was identified as a risk factor for PPID [1,40,41], which may explain that horses in the PPID group were significantly older than in the nPPID group. A frequently reported sign of ageing horses, loss of muscle tone, was reported by owners of geriatric horses [42,43], which was previously reported as a sign of ageing in horses [44]. Among hypertrichosis and/or other haircoat abnormalities, laminitis, lethargy, depression and weight loss, and epaxial muscle wastage or muscle atrophy are counted to be the most common clinical signs reported in horses suffering from

PPID [45,46]. These changes may also be responsible for some of the changes in the amino acid profile.

In human ACTH—secreting pituitary adenoma, changes in the amino acid metabolism have been reported; particularly, these concern the alanine, aspartate, and glutamate metabolism [47]. Significant differences in the plasma concentration of asparagine, the neutral derivative of aspartic acid, were also detected in horses suffering from PPID. Healthy horses and PPIDrr patients had significantly lower asparagine concentrations when compared to PPID patients. Furthermore, PPIDarr had significantly higher asparagine concentrations than healthy horses. Additionally, higher asparagine concentrations in horses suffering from PPID compared to healthy horses have already been reported previously [48]. However, the results for asparagine must be interpreted with care since 20 horses fell below the detection limit. Also, glutamine, which was significantly lower in plasma in healthy horses than in untreated PPID patients and PPID patients who received pergolide but whose ACTH concentration was above the reference range, is synthesized from glutamic acid and ammonia [49]. An altered activity of this metabolic pathway also seems conceivable in horses suffering from PPID, especially since epaxial muscle wasting is a typical sign of PPID [45,46] and glutamine is assumed to potentially be a direct regulator of muscle synthesis and degradation [50,51]. Therefore, it could be suggested that a higher glutamine concentration in PPID patients may be assumed, since stress leads to a release of high concentrations of glutamine [52,53]. However, the storages eventually become depleted [52,53], which may explain the lower glutamine concentrations in the PPID and PPIDarr groups.

Furthermore, decreased glutamic acid, arginine, cysteine, and glutamine levels were shown to be associated with oxidative stress and neurodegeneration in Parkinson's disease [28]. Since PPID is a neurodegenerative disease as well, decreased concentrations of these amino acids may also reflect the progression of the disease and might be used as potential markers of disease severity in the future.

The non-proteogenic amino acid citrulline was significantly higher in PPIDrr horses than in nPPID and PPID horses. This amino acid is mostly metabolized by the small intestine; therefore, it is considered to be a biomarker for the functional small intestinal bowel mass [54,55]. Furthermore, since citrulline is an intermediate metabolite in ureagenesis [56,57], during which citrulline is metabolized to arginine, a major regulator of vascular tone [58–60] in the kidney [61], it also is a functional biomarker for kidney function. In some cells, citrulline can act as a precursor for arginine [62] and, therefore, may be of importance for the metabolism and regulation of nitric oxide (NO) [63]. Regarding decreased citrulline concentrations and increased arginine concentrations, an upregulation of this pathway in the PPIDrr group seems conceivable; however, it should be clearly underlined again that only a few of these animals were included in the study. However, a previous study on gastrointestinally-diseased horses developing laminitis found significantly lower citrulline concentrations in horses developing laminitis than in those that did not [64]. Horses suffering from PPID are at risk for developing (endocrinopathic) laminitis [65]; however, endocrinopathic laminitis has a different pathogenesis than laminitis caused by gastrointestinal disease. Whether or not a decreased citrulline concentration is also a characteristic in equine endocrinopathic laminitis remains to be elucidated.

Cysteine is one of the least abundant, but functionally important, amino acids in proteins [66]. Mutations including cysteine residues include genetic diseases [67]. Cysteine molecules can react with other cysteine molecules by forming the typical disulfide bond, which can functionally interchange with another amino acid, selenocysteine, which is assumed to occur exclusively at functional sites [68]. A study performed on anterior pituitary cells in primary culture showed that cysteine proteases, in addition to aspartyl proteases, may be involved in the cellular metabolism of ACTH [69]. An association of these findings with lower cysteine concentrations due to altered ACTH production and pergolide treatment in PPIDrr patients remains to be elucidated, especially since these experiments were not conducted in horses but in cellular culture. Studies on human subjects with ACTH-secreting pituitary tumors also revealed metabolic changes: one study reported on

12 initially significantly changed metabolites in pituitary adenoma samples when compared to the control samples; after performing a Bonferroni correction, there were only three metabolites significantly changed: pyridoxate, deoxycholic acid, and 3-methyladipate [47]. Several changes were also detected in the metabolic pathway analysis, including changes in amino acid metabolism. Alanine, aspartate and glutamate metabolism, and starch and sucrose metabolism, as well as amino sugar and nucleotide sugar metabolism, lysine biosynthesis, vitamin B6 metabolism, aminoacyl-tRNA biosynthesis, glycolysis or gluconeogenesis, and purine metabolism, were significantly affected. The metabolism of alanine, aspartate, and glutamate was particularly affected [47]. Similarly, significant differences in the asparagine concentration, which is the neutral derivate of aspartate, were observed between the PPIDarr and the nPPID group. Different observations between the mentioned study and our study are probably caused by the different pathogenesis of the diseases.

5. Conclusions

Altered amino acid concentrations are found in horses suffering from PPID when compared to healthy horses. Especially asparagine, citrulline, and glutamine may be potential diagnostic markers and may offer interesting approaches for research regarding amino acid supplementation in PPID patients.

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4. Paper II - Plasma Amino Acid Concentration in Obese Horses with/without Insulin Dysregulation and Laminitis

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Plasma Amino Acid Concentration in Obese Horses with/without Insulin Dysregulation and Laminitis

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Simple Summary: Laminitic horses commonly suffer from an endocrine disease, such as equine metabolic syndrome (EMS). Hyperinsulinemia, which is in EMS patients caused by the inability to respond adequately to an oral carbohydrate load, is considered a key factor in the pathogenesis of laminitis. Since insulin also affects protein turnover in the body, the resting plasma amino acid concentrations of obese horses that were presented for a combined glucose insulin test (CGIT), which examines the insulin sensitivity of the tissues, were determined. In total, 25 obese horses and two lean horses with recurrent laminitis underwent a CGIT. Significant differences in the resting concentrations between obese and insulin dysregulated and laminitic (citrulline, GABA, methionine), as well as between insulin dysregulated individuals with and without laminitis (GABA) regarding three amino acids, were determined. This may be an interesting approach, especially for diagnostic testing and possibly also for the feed supplements of horses at risk of developing laminitis. However, further research, including a higher number of cases, is required.

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Abstract: Laminitic horses commonly suffer from an endocrine disease such as equine metabolic syndrome. Hyperinsulinemia is considered a key factor in the pathogenesis of laminitis. Since insulin also affects protein turnover in the body, the resting plasma amino acid concentrations of obese horses that were presented for a combined glucose insulin test (CGIT) were determined. In total, 25 obese horses and two lean horses with recurrent laminitis underwent a CGIT. Of these, five were not insulin dysregulated (obese), 14 were insulin dysregulated (ID), and eight were insulin-dysregulated and laminitic (IDL). Significant differences in the resting concentrations between obese and insulin dysregulated and laminitic (citrulline $p = 0.038$, obese: 73.001 ± 12.661 nmol/mL, IDL: 49.194 ± 15.486 nmol/mL; GABA $p = 0.02$, obese: 28.234 ± 3.885 nmol/mL, IDL: 16.697 ± 1.679 nmol/mL; methionine $p = 0.018$, obese: 28.691 ± 5.913 nmol/mL, IDL: 20.143 ± 3.09 nmol/mL) as well as between insulin dysregulated individuals with and without laminitis (GABA $p < 0.001$, ID: 28.169 ± 6.739 nmol/mL) regarding three amino acids were determined. This may be an interesting approach, especially for diagnostic testing and possibly also for the feed supplements of horses at risk of developing laminitis. However, further research, including a higher number of cases, is required.

Keywords: insulin resistance; equine metabolic syndrome; amino acid; GABA; citrulline; methionine

1. Introduction

The development of laminitis in horses is often associated with an endocrine disease (equine metabolic syndrome = EMS; pituitary pars intermedia dysfunction = PPID), in which insulin dysregulation occurs (EMS) or can occur (PPID) [1–3]. The suspected pathomechanism of endocrinopathic laminitis is hyperinsulinemia, but the mechanism by which hyperinsulinemia can cause laminitis remains unclear [4]. Based on the results of previous studies, it is suggested that insulin has an effect in the lamellar tissue of the

hoof via the insulin-like growth factor 1 receptor (IGF-1R) [5–7], and also that insulin can directly trigger laminitis even through mechanisms that have not yet been identified [4]. In addition to its effects on the glucose metabolism, insulin is an important regulator of protein metabolism. Due to an increased utilization of amino acids, hyperinsulinemia can contribute to an increased amino acid and protein turnover, for example in the skin or skeletal muscles [8–12]. In humans and dogs that are suffering from necrotic, wandering erythema and/or superficial necrolytic dermatitis, a reduced concentration of amino acids in the blood was found in some cases. After supplementation with amino acids the clinical picture improved or disappeared [13–16]. Since hoof horn is an appendix to the epidermis, the changes that occur in laminitis could also be influenced by an altered amino acid concentration. A recently published study in healthy horses showed that horses with euglycemic, hyperinsulinemic clamps for 48 h and glucose infusion for 66 h, had a reduction in blood plasma amino acids and the clinical signs of laminitis [4]. Furthermore, altered postprandial levels of citrulline, histidine, isoleucine, leucine, methionine, ornithine, tyrosine, and valine in horses with EMS, were described previously [17].

Therefore, the aim of our study was to identify differences in fasting amino acid concentrations in obese and insulin dysregulated horses with and without laminitis.

2. Materials and Methods

2.1. Study Participants

For this prospective study, adult horses and ponies (>3 years old, horses and ponies are both referred to as "horses") that presented to the Equine Clinic: Surgery and Radiology of the Freie Universität, Berlin, for a combined glucose-insulin test (CGIT) and radiographic evaluation of the distal phalanges (front limbs or both hind and front limbs), were considered for the study. Except for the obvious obesity, the clinical examination of these horses was within normal limits. None of the horses were lame at walk or showed pain at turning. Recorded data for each patient included age, breed, gender, feeding, treatment with pergolide as well as the body condition score (BCS) [18,19]. As confirmed by previous PPID diagnosis, an increased ACTH concentration depending on season and/or a positive TRH stimulation test was defined. Horses were defined as insulin dysregulated/non-insulin dysregulated according to the results of the dynamic tests (see Section 2.2 Dynamic Testing).

The radiographs of the distal phalanges were evaluated by a specialist for equine medicine (Fachtierärztin für Pferde, SDS). Horses were defined as (chronically) laminitic if there was rotation or ventral displacement of the distal phalanx, as reviewed by Thieme et al. [20].

2.2. Dynamic Testing

The horses were stabled in the evening before the day on which the CGIT was performed. On the same day as the test was performed, they received an intravenous catheter before starting the CGIT. All horses tolerated the placement of the catheter well, none of them was insubordinate or required sedation. Before dynamic testing, the horses were fasted for 6 h.

The CGIT was performed as described by Eiler et al. [21]. Basal concentrations of glucose and insulin were determined and a sample for endogenous ACTH measurement, as well as amino acid determination collected. For insulin determination, blood was collected in serum tubes (Sarstedt, Nümbrecht, Germany), and for ACTH measurement, in EDTA tubes (Sarstedt, Nümbrecht, Germany). Within 15 min of collection, the blood samples for ACTH measurement were centrifuged and the samples for insulin concentration were centrifuged within 30 min of collection (bot 2000× *g* for 10 min). Plasma and serum were transported cooled to the external laboratory.

Samples for plasma glucose concentration were collected in a blood collection syringe (BD A-Line, Becton Dickinson AG, Basel, Suisse) and directly evaluated with an automated clinical blood gas analyzer (Cobas b 123, Roche Deutschland Holding GmbH, Grenzach-Wyhlen, Germany).

Then, 150 mg/kg Glucose (Glucose 40% ad us vet., B. Braun Vet., Melsungen, Germany) and 0.1 U insulin/kg bodyweight (Caninsulin® 40 IU/mL, Intervet Deutschland GmbH, Unterschleißheim, Germany) were administered intravenously. Samples for glucose concentration determination were collected at 1, 5, 15, 25, 35, 45, 60, 75, 90, 105, 120, 135, and 150 min. At 45 min, a sample for the stimulated insulin concentration was also collected. If the glucose concentration had returned to the baseline before completion of the 150 min, the test was terminated. Horses were defined as insulin dysregulated if the glucose concentration did not return to baseline within 45 min after glucose and insulin administration, and/or the insulin concentration was greater than 100 µU/mL at the same time point (45 min).

The ACTH and insulin concentrations were determined by Laboklin (Laboklin GmbH and Co. KG, Bad Kissingen, Germany).

2.3. Determination of the Amino Acid Concentration

Blood for the amino acid analysis was collected with the baseline samples of the CGIT in EDTA tubes (Sarstedt, Nümbrecht, Germany). Within 15 min of collection, the samples were centrifuged at 2000× *g* for 10 min and the blood plasma frozen at −20 °C for the amino acid analysis.

2.3.1. Sample Preparation for the Amino Acid Analysis

The samples were prepared by mixing 400 µL plasma and 400 µL sample dilution solution (Lithium Loading Buffer Kit, Biochrom Ltd., Cambridge, UK) which included the internal standard Norleucine 200 nmol/mL) in an Eppendorf tube. To this, 200 µL 10% (*w/v*) 5-sulfosalicylic acid (SSA) solution for the deproteinization were added and the sample was deposited in the refrigerator for 20 min at 4 °C. Afterwards, the sample was centrifuged (Eppendorf centrifuge 5415 C) at 11,000× *g* for 5 min. The supernatant was centrifuged a second time with a nylon membrane filter, pore size 0.22 µm (Laborservice Onken, Gruendau, Germany) at 11,000× *g* for 2 min.

2.3.2. Amino Acid Analysis

For the quantitative analysis, the free amino acids were determined with the Biochrom 30+ amino acid analyzer (Harvard Bioscience, Holliston, MA, USA). The amino acid analysis method is based on ion exchange chromatography with post column derivatization with Ninhydrin (Lithium Buffer 1-6 Kit and NZ-Ninhydrin Reagent Kit, Biochrom Ltd., Cambridge, UK).

The physiological standard sample (amino acid physiological standard solution, 40 amino acids, Laborservice Onken, Gründau, Germany) with known amino acid concentrations 200 nmol/mL standard was compared with the horse plasma samples using the EZ Chrome elite software (Agilent, Santa Clara, CA, USA).

2.4. Statistical Analysis

The statistical analysis was conducted with the IBM® SPSS® Statistics Version 28.0.1 (IBM Deutschland GmbH, Ehningen/Germany).

The horses were assigned to the following groups: obese, but non-insulin dysregulated and non-laminitic (obese); insulin-dysregulated and non-laminitic (ID); and insulin dysregulated and laminitic. (IDL). Before performing statistical tests, the data were tested for normal distribution.

The means of the normally distributed data were compared using a one-way ANOVA and in case of no homogeneity of variance the Welch test, for non-normally distributed data, the Kruskal-Wallis test was used. For post-hoc testing, the Tukey and the Games-Howell Test were employed.

2.5. Ethical Statement

The study was not declared according to the German Animal Welfare law §8.1 since all samples were taken as a part of a routine clinical examination. Written owners' consent to involve their horses in the study was obtained during the admission process at the clinic.

3. Results

3.1. Study Participants

In total, 12 mares and 15 geldings suspected to suffer from insulin dysregulation were presented to the clinic. Two of the equids were not obese but were tested due to recurrent laminitis. One of these two horses was also treated with pergolide. Of the 27 horses presented for dynamic testing, five were not insulin dysregulated (obese), 14 were insulin dysregulated (ID), and eight were insulin-dysregulated and laminitic (IDL). All laminitic horses were insulin dysregulated.

Included equids were mostly ponies; only five were of other breed (Appaloosa (2), Arabian cross, Warmblood, Andalusian horse).

The equids were aged 3–22 years, and horses being IDL (16.63 +/− 2.32 years) were significantly older than obese individuals (9.8 +/− 2.95 years, $p = 0.008$, Games Howell Test). There was no significant difference regarding weight (345.93 +/− 155.28 kg, $p = 0.716$, ANOVA), BCS (8.04 +/− 0.76, $p = 0.104$), and gender ($p = 0.103$) between the groups. All horses, except for one (IDL), that also received a mineral supplement, were kept on a hay diet exclusively. Except for one horse that received pergolide (IDL) the horses did not receive any medications. Demographic data of the study groups are displayed in Table 1.

Table 1. Demographic data of study participants (mean +/− standard deviation or absolute numbers of horses).

	Obese	Insulin Dysregulated	Insulin Dysregulated and Laminitic
Age (years)	9.80 ± 2.95	14.14 ± 5.11	16.63 ± 2.32
Weight (kg)	294.0 ± 167.6	353.6 ± 137.1	365.0 ± 190.2
Body condition score	8.0 ± 0.0	8.3 ± 0.5	7.6 ± 1.1
Previous diagnosis of PPID	0	0	1
Treatment with pergolide	0	0	1

3.2. Endocrine Testing

There was no significant difference in the baseline blood glucose concentration between the groups ($p = 0.507$, ANOVA), but ID and IDL horses required significantly more time for the blood glucose concentration to return to baseline than obese horses (obese: 35.0 +/− 10 min, ID: 121.3 +/− 31.6 min, IDL: 150.63 +/− 0.5 min; obese vs ID $p < 0.001$, obese vs. IDL $p < 0.001$, Tukey test). Furthermore, the blood glucose concentration was significantly longer elevated in IDL than in ID horses ($p = 0.026$, Tukey test).

The resting insulin concentration was significantly higher in ID (7.08 +/− 3.75 µU/mL) and IDL (15.24 +/− 9.38 µU/mL) horses than in obese horses (obese: 2.5 +/− 0.93 µU/mL; obese vs. ID $p = 0.002$, obese vs. IDL $p = 0.015$, Games Howell test). The stimulated insulin concentration was unavailable for three horses: in two obese horses, the test was cancelled after 25 min since the baseline blood glucose concentration was reached and in one IDL horse the sample was lost on the way to the laboratory. The stimulated insulin concentration was significantly higher in ID (51.83 +/− 22.49 µU/mL $p = 0.011$, Games Howell test) and IDL (144.73 +/− 84.6 µU/mL, $p = 0.017$ Games Howell Test) than in obese horses (18.31 +/− 9.93 µU/mL), but there was no significant difference between ID and IDL ($p = 0.061$, Games Howell test). There was no significant difference regarding the ACTH concentration between the groups (Kruskal Wallis test), but three horses (2 IDL, 1 obese) had resting ACTH concentrations above 100 pg/mL. These horses were not treated

with pergolide. Concentrations of ACTH and insulin of the study groups are displayed in Table 2.

Table 2. ACTH and insulin concentration in obese, insulin dysregulated, and insulin dysregulated laminitic horses (median (minimum-maximum) or mean +/− standard deviation).

	Obese	Insulin Dysregulated	Insulin Dysregulated and Laminitic
ACTH (<30 pg/mL)	19 (12.5–196)	21.9 (10.1–80.8)	16.7 (9.8–963)
Insulin at 0 min (<20 µU/mL)	2.5 +/− 0.93 µU/mL;	7.08 +/− 3.75 µU/mL	15.24 +/− 9.38 µU/mL
Insulin at 45 min (<100 µU/mL)	18.31 +/− 9.93 µU/mL	51.83 +/− 22.49 µU/ml	144.73 +/− 84.6 µU/mL

3.3. Amino Acid Concentrations

Mean and standard deviations of the normally distributed amino acid concentrations as well as median, maximum and minimum of the non-normally distributed amino acid concentrations are displayed in Tables 3 and 4. Furthermore, the count of available samples and the *p*-value were included into the tables as well.

Table 3. Mean and standard deviations of the normally distributed amino acid concentrations (nmol/mL).

Amino Acid		Obese	ID	IDL	*p* (ANOVA)
1-Methyl-Histidine	Mean	23.986	19.414	17.147	0.335
	Standard deviation	10.613	8.663	5.062	
	Available Samples	27	27	27	
Alanine	Mean	206.175	226.394	223.001	0.789
	Standard deviation	69.407	46.584	64.663	
	Available Samples	27	27	27	
Arginine	Mean	73.388	65.889	69.635	0.772
	Standard deviation	18.05	17.323	20.641	
	Available Samples	27	27	27	
Asparagine	Mean	19,858	18,167	20,147	0.726
	Standard deviation	7.009	4.08	8.183	
	Available Samples	27	27	27	
Citrulline	Mean	73.001	53.724	49.194	0.038
	Standard deviation	12.661	16.95	15.486	
	Available Samples	27	27	27	
GABA (Gamma-aminobutyric acid)	Mean	28.234	28.169	16.697	<0.001
	Standard deviation	3.885	6.739	1.679	
	Available Samples	27	27	27	
Glutamine	Mean	263.382	254.664	237.805	0.728
	Standard deviation	61.853	50.585	74.643	
	Available Samples	27	27	27	

Table 3. *Cont.*

Amino Acid		Obese	ID	IDL	*p* (ANOVA)
Glutamic acid	Mean	16.292	18.53	25.391	0.116
	Standard deviation	2481	6956	12,126	
	Available Samples	27	27	27	
Glycine	Mean	43.641	393.742	369.839	0.669
	Standard deviation	84.764	152.069	10.065	
	Available Samples	27	27	27	
Histidine	Mean	73.297	76.231	71.124	0.314
	Standard deviation	10.55	7.2	5.819	
	Available Samples	27	27	27	
Lysine	Mean	70.1	68.014	80.389	0.363
	Standard deviation	17.088	21.831	15.858	
	Available Samples	27	27	27	
Methionine	Mean	28.691	25.617	20.143	**0.019**
	Standard deviation	5.913	4.862	3.09	
	Available Samples	27	27	25	
Ornithine	Mean	48.718	49.153	48.782	0.997
	Standard deviation	10.915	15.553	11.795	
	Available Samples	27	27	27	
Phenylalanine	Mean	52.286	53.543	56.846	0.491
	Standard deviation	12.96	6.437	3.833	
	Available Samples	27	27	27	
Proline	Mean	61.044	57.062	61.151	0.834
	Standard deviation	20.69	10.817	23.946	
	Available Samples	27	27	27	
Serine	Mean	181.459	214.725	229.933	0.420
	Standard deviation	45.655	66.886	66.504	
	Available Samples	27	27	27	
Taurine	Mean	39576	36.345	33.037	0.674
	Standard deviation	5.812	15.998	9.218	
	Available Samples	27	27	27	
Tryptophan	Mean	64.037	64.699	72.756	0.308
	Standard deviation	11.293	12.67	12.661	
	Available Samples	27	27	27	
Valine	Mean	160.249	163.951	190.369	0.274
	Standard deviation	24.689	39.447	46.052	
	Available Samples	27	27	27	

With ANOVA, significant group differences were identified for citrulline ($p = 0.038$), gamma-aminobutyric acid (GABA, $p < 0.001$), and methionine ($p = 0.019$). For post-hoc testing, the Tukey and the Games Howell tests were employed.

The plasma citrulline concentration was significantly lower in IDL than in obese horses ($p = 0.038$), there was no significant difference between obese and ID ($p = 0.071$) as well as ID and IDL ($p = 0.789$).

Table 4. Median, minimum, and maximum of the non-normally distributed amino acid concentrations (nmol/mL).

Amino Acid		Obese	ID	IDL	*p* (Kruskal Wallis Test)
Isoleucine	Median	44.325	59.348	65.526	0.084
	Maximum	66.544	100.782	84.085	
	Minimum	41.413	35.487	57.173	
	Available Samples	27	27	26	
Leucine	Median	77.476	98.757	120.116	0.078
	Maximum	128.293	123.473	143,189	
	Minimum	71.808	69.43	60.7	
	Available Samples	27	27	27	
Threonine	Median	117.291	81.244	67.396	0.59
	Maximum	149.826	170.211	212.1	
	Minimum	50.631	58.425	39.823	
	Available Samples	27	27	27	
Tyrosine	Median	70.656	62.53	58.03	0.806
	Maximum	76.974	74.186	84.769	
	Minimum	35.127	43.782	50.1	
	Available Samples	27	27	27	

The plasma GABA concentration was significantly higher in obese ($p = 0.002$) and ID horses ($p < 0.001$) when compared to the IDL group. However, there was no significant difference between the obese and ID group ($p = 1$).

Methionine was significantly higher concentrated in the plasma of obese individuals when compared to IDL patients ($p = 0.018$). Significant differences between obese and horses suffering from ID ($p = 0.441$) as well as between the ID and IDL group ($p = 0.067$) were not detected.

4. Discussion

This study describes the resting amino acid concentration of 25 obese horses and two lean horses with recurrent laminitis that underwent a CGIT. Of these 27 horses, five were not insulin dysregulated (obese), 14 were insulin dysregulated (ID), and eight were insulin-dysregulated and laminitic (IDL). Significant differences in the resting concentrations between obese and insulin dysregulated and laminitic (citrulline $p = 0.038$, obese: 73.001 $\pm$ 12.661 nmol/mL, IDL: 49.194 $\pm$ 15.486 nmol/mL; GABA $p = 0.02$, obese: 28.234 $\pm$ 3.885 nmol/mL, IDL: 16.697 $\pm$ 1.679 nmol/mL; methionine $p = 0.018$, obese: 28.691 $\pm$ 5.913 nmol/mL, IDL: 20.143 $\pm$ 3.09 nmol/mL) as well as between insulin dysregulated individuals with and without laminitis (GABA $p < 0.001$, ID: 28.169 $\pm$ 6.739 nmol/mL) regarding three amino acids were determined.

The plasma amino acid profile may be affected by the type of feeding the horses receive. All horses were exclusively fed with hay except for one that received a mineral supplement additionally. However, differences in the nutrient content of hay were reported previously [22,23]. Since all horses were kept off feed for six hours prior to testing, the influence of the feeding is not considered as relevant.

Additionally, three horses had laboratory signs for pituitary pars intermedia dysfunction (PPID) in which the increased endogenous glucocorticoid concentrations are known to be associated with systemic insulin resistance [24–29] and thus may have influenced the amino acid profile additionally [30,31]. One further horse was treated with pergolide which may have additionally influenced the concentrations of the amino acids [30].

An optimal study would have compared larger groups of horses that were kept under the same conditions for a few weeks and that did not show signs of additional endocrinopathies.

Stokes et al. reported significant changes of the plasma amino acid concentration in an experimentally produced hyperinsulinemia [4]. However, they found slightly different changes during the euglycemic hyperinsulinemic clamp compared to the prolonged glucose infusion [4]. Furthermore, they found a significant decrease in 15 (prolonged glucose infusion) and 19 (euglycemic hyperinsulinemic clamp) of the 20 amino acids determined in the plasma [4], whereas in this study, only three amino acids (citrulline, GABA, methionine), of which not all are proteogenic, had significantly different concentrations between obese and insulin dysregulated and laminitic (citrulline, GABA, methionine) as well as between insulin dysregulated individuals with and without laminitis (GABA). This may be attributed to the fact, that resting amino acid concentrations were measured and none of the horses had a resting hyperinsulinemia. However, in both the euglycemic hyperinsulinemic clamp and the prolonged glucose infusion, the concentration of methionine was one of the amino acids that showed a marked decrease during hyperinsulinemia [4] and methionine was also one of the amino acids that showed a significant difference between insulin dysregulated non-laminitic and laminitic animals. Since none of the horses showed a resting hyperinsulinemia, less changes were observed in this study. Contrasting to this, higher postprandial concentrations of citrulline and methionine after a high protein meal were described in horses suffering from EMS when compared to healthy horses [17]. It may have been interesting to examine the amino acid concentrations after 45 min, at least in this cohort with marked increases in the insulin concentration after stimulation.

Methionine, the amino acid that showed marked decreases during induced hyperinsulinemia [4] and was significantly less concentrated in the plasma of insulin dysregulated and laminitic horses than in the plasma of obese horses, is an essential proteinogenic amino acid [32]. Furthermore, methionine as well as cysteine are a part of the glutathione metabolism, which is the major antioxidant in mammalian cells [33]. In humans, a nutritional methionine deficit was associated with diseases such as toxemia, childhood rheumatic fever, muscle paralysis, hair loss, depression, schizophrenia, Parkinson's disease, liver deterioration, and impaired growth [34]. In healthy lactating cows, supplementation with methionine led to an increase hoof horn growth rate, but also to alterations of the amino acid profile of the hoof horn. Supplemented cows had less cysteine and proline but greater percentages of methionine, lysine, tyrosine, and glutamic acid levels in their hoof horn. The authors suggested that this may be caused by a decrease of disulfide bonding in the hoof tissues [35]. Another study in heifers during the first 13 weeks of lactation found increased growth rates but unchanged wear rates during methionine supplementation which led to conformational changes of the hoof [36]. Other authors reported a significant decline of heel erosions, sole avulsions, and the resolution of all the white line hemorrhages [37]. However, methionine was claimed to be the most toxic amino acid in relation to growth in animals [38]. Contrasting to this, a review on methionine toxicity in humans concluded that serious methionine toxicity only occurs only at very high levels of intake [39]. In animals, methionine and cysteine toxicity were associated with consumption at levels five times greater than required [40] and the free sulfhydryl group of cysteine was assumed to be the cause of toxicity in chicks [41]. However, the mechanism of toxicity is not completely understood [42]. A study examining methionine supplementation in weanling Quarter horses (basal: 0.2% methionine, basal + 0.03% methionine: 0.23% methionine, basal + 0.07% methionine: 0.27 methionine, basal + 0.11% methionine: 0.3% methionine) suggested that the methionine requirements for growing Quarter horses may fall between 0.23% and 0.31% methionine [42]. Requirements for insulin-dysregulated horses have

not been published so far. This may be interesting for further research especially since beside Stokes et al. [4] and a study by Kenéz and colleagues identified significantly lower methionine in addition to lower trans–4 hydroxyproline levels in insulin-dysregulated, compared to insulin–sensitive horses [43].

GABA is a non-proteogenic amino acid which acts as an inhibitory neurotransmitter in mammalian neural tissues [44]. However, recent research on wound healing in rats suggests an anti-inflammatory and fibroblast proliferation stimulating role of GABA. In this study, GABA treatment was effective in accelerating the healing process [45]. Additionally, a study in humans identified an improvement of the skin elasticity in humans of GABA by regulating type I collagen expression [46]. As commonly known, hoof horn is the appendix to the epidermis, at which the damage during endocrinopathic laminitis occurs. In the case of a GABA deficiency during hyperinsulinemia, the healing of the damage that occurred during hyperinsulinemia might be delayed. This seems to be an interesting subject for future studies. As GABA, citrulline is a non-proteogenic amino acid as well, which acts as an intermediate in the metabolite in the ureagenesis [47,48]. Since citrulline is almost exclusively metabolized by the small intestine, the plasma citrulline concentration is considered as a biomarker of the functional small intestinal bowel mass [49,50]. Furthermore, citrulline is a functional biomarker in renal failure since the kidney is the only organ that metabolizes citrulline into arginine [51], which is a major regulator of vascular tone [52–54]. Since in some cells arginine can be recycled from citrulline, it can act as a precursor for arginine [55] and may therefore be of importance for the nitric oxide (NO) metabolism and regulation [56]. NO-mediated vasodilation and GLUT 4 translocation are initiated by stimulation of insulin receptors in healthy horses [57]. In insulin resistance, this pathway may be blocked and therefore the alternative mitogen-activated protein kinase pathway activated. This leads to endothelin 1-mediated vasoconstriction, the upregulation of cellular adhesion molecules, and mitogenesis [57]. In this study, insulin dysregulated laminitic horses had significantly lower citrulline concentrations than obese animals. Furthermore, a study focusing on the citrulline concentration in horses with gastrointestinal disease found lower citrulline concentrations in horses developing laminitis compared to those who did not [58]. The clinical relevance of this finding remains to be elucidated. Clearly, further studies are required to examine amino acid concentrations as a marker in equine endocrinopathic disease, or as a potential supplement for (previously) laminitic horses.

5. Conclusions

There are different resting concentrations between obese and insulin dysregulated and laminitic (citrulline, GABA, methionine) as well as between insulin dysregulated individuals with and without laminitis (GABA). This may be an interesting approach especially for diagnostic testing and possibly also for feed supplements of horses at risk of developing laminitis. However, further research including a higher number of cases is required.

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Institutional Review Board Statement: The study was not declared according to the German Animal Welfare law §8.1 since all samples were taken as a part of a routine clinical examination. According to §6 of the German Animal Welfare Law blood collection from animals is allowed if there is a veterinary indication. All animals had a medical indication for blood collection (diagnostic testing for insulin dysregulation, all animals were obese or lean with recurrent laminitis)-no additional blood was collected. Written owner's consent to involve their horses in the study was obtained during the admission process at the clinic. The owners consented to their horse(s) being presented in clinical

lectures and being examined by veterinary students under the supervision of a veterinarian employed by the clinic. Furthermore, they consented to further evaluation of data and sample materials collected as part of the regular examination for scientific purposes and anonymous publication.

Informed Consent Statement: Written owner's consent to involve their horses in the study was obtained during the admission process at the clinic. The owners consented to their horse(s) being presented in clinical lectures and being examined by veterinary students under the supervision of a veterinarian employed by the clinic. Furthermore, they consented to further evaluation of data and sample materials collected as part of the regular examination for scientific purposes and anonymous publication.

Data Availability Statement: Additional data can be obtained upon request from the corresponding author.

Conflicts of Interest: Detlef Timmermann, who was involved in the investigation, data curation and writing (review and editing), is an employee of Laborservice Onken GmbH. All authors declare no conflict of interest with regard to the writing of this manuscript.

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5. Discussion

Plasma amino acid profile

In both parts of this project, differences of the plasma amino acid profile between horses suffering from endocrine disease and healthy/obese horses were shown.

As in these horses, changes in the metabolome were described previously in humans with endocrine disease. For example, changes in amino acid metabolism were detected in human ACTH-secreting pituitary adenoma. These affected in particular the alanine, aspartate and glutamate metabolism (Oklu et al. 2014). Significant differences in the plasma concentration of asparagine, the neutral derivative of aspartic acid, were also detected in horses suffering from PPID whose ACTH concentration was above the reference range. Healthy horses and treated PPID patients with ACTH concentrations in the reference range had significantly lower asparagine concentrations compared to PPID patients. Furthermore, treated PPID patients with ACTH concentrations above the reference range had significantly higher asparagine concentrations than healthy horses. In addition, higher asparagine concentrations have been reported in horses with PPID compared to healthy horses (De Vries 2015). Glutamine, which was significantly less concentrated in plasma in healthy horses than in untreated PPID patients and PPID patients that received pergolide but whose ACTH concentration was above the reference range, is synthesised from glutamic acid and ammonia (Krebs 1935). An altered activity of this metabolic pathway therefore also seems conceivable in horses suffering from PPID. Furthermore, epaxial muscle wasting is a typical sign of PPID (Frank et al. 2006; Hart et al. 2021) and glutamine is assumed to be potentially a direct regulator of muscle synthesis and degradation (Maclennan et al. 1987; Maclennan et al. 1988). Therefore, one could expect a higher glutamine concentration in PPID patients, since stress leads to a release of high concentrations of glutamine (Lacey and Wilmore 1990; Souba 1991).

Changes in the metabolome also occur in humans in diseases of other organ systems. Parkinson's disease, like PPID, is associated with oxidative damage to dopaminergic nerve pathways (McFarlane 2007).

Decreased glutamic acid, arginine, cysteine and glutamine levels were shown to be associated with oxidative stress and neurodegeneration in Parkinson’s disease (Figura et al. 2018). Since PPID is a neurodegenerative disease as well, decreased concentrations of these amino acids may also reflect the progression of the disease and might be used as potential markers of disease severity in the future. This is underlined by treated animals whose ACTH concentration was above the reference range having significantly higher plasma arginine concentrations than healthy horses.

Furthermore, there were also differences in the amino acid profile with regard to the development of complications in horses with diseases of the gastrointestinal tract. Thus,

citrulline concentration was significantly lower in patients with gastrointestinal diseases who developed laminitis than in those who did not develop laminitis during treatment (Jackson 2013). Based on this project and the project described in the thesis, plasma citrulline concentration could be an interesting biomarker to detect horses at risk of laminitis. In addition to plasma citrulline concentration, plasma methionine concentration also seems to have potential as a biomarker for the detection of individuals at risk of laminitis: Additionally to the projects presented here, a lower plasma methionine concentration was described in horses with insulin-dysregulation previously (Kenéz et al. 2018). Furthermore, a decrease in methionine concentration was also observed in experimentally induced hyperinsulinaemia (Stokes et al. 2021).

This project, which addresses two most common equine endocrinopathies, EMS and PPID, in terms of amino acid profile, shows that endocrinopathies in horses can cause a change in the plasma amino acid profile. However, the different changes also show that these are also influenced by the pathogenesis of the endocrinopathy as well as its consequences and, if necessary, its treatment. Unfortunately, it was not possible to examine all PPID affected horses and their healthy controls radiographically, so that no reliable conclusion can be made about the presence of subclinical laminitis and thus statements about potential changes in PPID patients suffering from chronic laminitis are not possible based on this project. However, since hyperinsulinaemia is suspected to be the underlying pathomechanism of endocrinopathic laminitis (De Laat et al. 2013; Lane et al. 2017; Sandow et al. 2019), it seems possible that patients suffering from chronic laminitis also show further changes in the plasma amino acid profile.

This is also supported by a study on Holstein bulls suffering from nutritionally induced laminitis. Lower concentrations of phosphatidylcholines and sphingomyelines and higher concentrations of lyso-phosphatidylcholines, branched-chain amino acids and aromatic amino acids were found than in animals not suffering from laminitis (Bäßler et al. 2021).

Overall, it therefore seems conceivable that modified feeding/supplementation of certain amino acids might be useful for the prevention of an acute episode of chronic laminitis.

The aim of further research should therefore be, on the one hand, to investigate and possibly establish the usefulness of citrulline and methionine as biomarkers for the detection of individuals at risk of laminitis and, on the other hand, to observe the effect of supplementation of these amino acids in corresponding individuals.

6. Summary

Effects of endocrinopathies on the plasma amino acid profile of horses

Horses suffering from laminitis usually have an underlying endocrine disease such as equine metabolic syndrome or a dysfunction of the pituitary gland (pitiutary pars intermedia dysfunction = PPID). Hyperinsulinaemia, which is caused by insulin-dysregulation, is currently assumed to be the triggering factor for laminitis. Insulin-dysregulation is present in equine metabolic syndrome and can also occur in horses suffering from PPID. It has already been shown that experimentally induced hyperinsulinaemia causes a change in the plasma amino acid profile of horses.

This project was divided into 2 sub-projects. In the first sub-project, the plasma amino acid concentration of horses suffering from PPID was compared with that of healthy horses. Therefore, EDTA-plasma of horses from horses that were presented at the Equine Clinic of the Freie Universität Berlin for different reasons and required laboratory tests of blood anticoagulated with EDTA was asserved. This plasma was used to determine the basal ACTH concentration as well as the amino acid profile. Horses were considered to have PPID if the ACTH concentration was ≥ 100 pg/ml, i.e. they were considered to have PPID at any time. Horses were defined as healthy (nPPID) if the ACTH concentration was below 30 pg/ml, so they were considered healthy at any time point. Horses that received pergolide and whose ACTH concentration was ≤ 30 pg/ml were assigned to the PPIDrr group (PPID, ACTH in the reference range) and horses that received pergolide and whose ACTH concentration was ACTH ≥ 100 pg/ml were assigned to the PPIDarr group (PPID, ACTH above the reference range). A total of 93 horses were examined, 88 of them in the clinic and 5 in a private practice. Of these, 53 horses met the inclusion criteria (ACTH ≤ 30 pg/ml or ACTH ≥ 100 pg/ml). 25 horses were diagnosed as nPPID, 20 as PPID, 5 as PPIDrr and 3 as PPIDarr. No significant differences were present between the groups in terms of sex and breed. However, significant differences were found between groups with respect to feeding, age and ACTH concentration. Significant differences were present between nPPID and PPIDrr and PPID and PPIDrr with respect to feeding: All horses identified as PPIDrr were fed hay only, whereas the nPPID and PPID patients were fed concentrates (1 nPPID), mash (10 nPPID, 3 PPID) or grass (1 nPPID, 12 PPID) in addition to hay. One horse (nPPID) received only grass. In addition, horses suffering from PPID were significantly older than nPPID horses. There were no significant differences in age between the other groups. There were no significant differences in ACTH concentration between nPPID and PPIDrr. The ACTH concentration of PPID patients was significantly higher than in nPPID, PPIDrr and PPIDarr. In addition, PPIDarr horses had significantly higher ACTH concentrations than PPIDrr horses. The following significant differences were present in plasma amino acid concentrations: Arginine was significantly

higher in PPIDrr than in PPID and nPPID, asparagine was significantly higher in PPID, PPIDrr and PPIDarr than in nPPID, citrulline was significantly higher in PPIDrr than in nPPID and PPID, cysteine was significantly lower in PPIDrr than in PPID, nPPID and PPIDarr and glutamine was significantly higher in PPID and PPIDarr than in nPPID.

In the second part of the project, the plasma amino acid concentration of obese horses with and without chronic laminitis/lean horses suffering from chronic laminitis presented for a combined glucose-insulin-test (CGIT) as well as for a radiographic examination of the hooves. A total of 25 obese horses and two lean horses with recurrent laminitis underwent CGIT. Of the 27 horses that presented, five were non-insulin-dysregulated (obese), 14 were insulin-dysregulated and eight were insulin-dysregulated with radiographic evidence of chronic laminitis. Significant differences in resting amino acid concentrations were found between obese and insulin-dysregulated laminitis affected horses (citrulline, GABA, methionine). Furthermore, a significant difference between insulin-dysregulated horses with and without laminitis was detected with respect to the plasma GABA concentration.
Overall, this project provides interesting approaches to develop further diagnostic tests, especially for the detection of horses at risk of laminitis, and possibly also for research into feed supplements.

7. Zusammenfassung

Auswirkungen von Endokrinopathien auf das Plasma-Aminosäureprofil von Pferden

Bei an Hufrehe leidenden Pferden liegt häufig eine endokrine Grunderkrankung wie beispielsweise das equine metabolische Syndrom oder eine Dysfunktion des Hypophysenzwischenlappens (Pitiutary Pars Intermedia Dysfunction = PPID) vor. Als auslösender Faktor für die Hufrehe wird aktuell eine Hyperinsulinämie, die durch eine Insulindysregulation zustande kommt, angenommen. Eine Insulindysregulation liegt beim equinen metabolischen Syndrom vor und kann auch bei an PPID erkrankten Pferden vorkommen. Es wurde bereits gezeigt, dass eine experimentell induzierte Hyperinsulinämie eine Veränderung des Plasma-Aminosäureprofils von Pferden bewirkt.

Das Projekt untergliederte sich in 2 Teilprojekte. Im ersten Teilprojekt wurde die Plasma-Aminosäurekonzentration von an PPID erkrankten Pferden mit der gesunder Pferde verglichen. Es wurde daher EDTA-Plasma von Pferden von Pferden, die aus unterschiedlichen Gründen in der Pferdeklinik der Freien Universität Berlin vorgestellt wurden und Laboruntersuchungen von mit EDTA antikoaguliertem Blut erforderten, asserviert. Mit diesem Plasma wurde die basale ACTH-Konzentration sowie das Aminosäureprofil bestimmt. Pferde galten als an PPID erkrankt, wenn die ACTH-Konzentration ≥ 100 pg/ml lag, sie also zu jedem Zeitpunkt als erkrankt gelten. Pferde wurden als gesund (nPPID) definiert, wenn die ACTH-Konzentration unter 30 pg/ml lag, sie also zu jedem Zeitpunkt als gesund gelten. Pferde, die Pergolid erhielten und deren ACTH Konzentration ≤ 30 pg/ml lag, wurden der Gruppe PPIDrr (PPID, ACTH im Referenzbereich) zugeordnet und Pferde, die Pergolid erhielten und deren ACTH Konzentration ACTH ≥ 100 pg/ml lag, wurden der Gruppe PPIDarr (PPID, ACTH oberhalb des Referenzbereichs) zugeordnet. Insgesamt wurden 93 Pferde untersucht, davon 88 Pferde in der Klinik und 5 Pferde in einer Privatpraxis. Davon erfüllten 53 Pferde die Einschlusskriterien (ACTH ≤ 30 pg/ml oder ACTH ≥ 100 pg/ml). 25 Pferde wurden als nPPID, 20 als PPID, 5 als PPIDrr und 3 als PPIDarr diagnostiziert. Zwischen den Gruppen lagen keine signifikanten Unterschiede hinsichtlich des Geschlechtes und der Rasse vor. Es wurden jedoch signifikante Unterschiede zwischen den Gruppen hinsichtlich der Fütterung, des Alters und der ACTH-Konzentration festgestellt. Zwischen nPPID und PPIDrr sowie PPID und PPIDrr lagen signifikante Unterschiede bezüglich der Fütterung vor: Alle als PPIDrr identifizierten Pferde wurden ausschließlich mit Heu gefüttert, während die nPPID- und PPID-Patienten neben Heu auch Kraftfutter (1 nPPID), Mash (10 nPPID, 3 PPID) oder Gras (1 nPPID, 12 PPID) erhielten. Ein Pferd (nPPID) erhielt nur Gras. Zudem waren an PPID erkrankte Pferde signifikant älter als nPPID-Pferde. Zwischen den anderen Gruppen gab es keine signifikanten Unterschiede hinsichtlich des Alters. Es gab keine signifikanten Unterschiede in der ACTH-Konzentration zwischen nPPID und PPIDrr. Die ACTH-Konzentration von PPID-Patienten war

signifikant höher als bei nPPID, PPIDrr und PPIDarr. Darüber hinaus hatten PPIDarr-Pferde signifikant höhere ACTH-Konzentrationen als PPIDrr-Pferde. Bei den Plasma-Aminosäurekonzentrationen lagen folgende signifikante Unterschiede vor: Arginin war signifikant höher bei PPIDrr als bei PPID und nPPID, Asparagin war signifikant höher bei PPID, PPIDrr und PPIDarr als bei nPPID, Citrullin war signifikant höher bei PPIDrr als in nPPID und PPID, Cystein war signifikant niedriger bei PPIDrr als bei PPID, nPPID und PPIDarr und Glutamin war signifikant höher bei PPID und PPIDarr als bei nPPID.

Im zweiten Teil des Projektes wurde die Plasma-Aminosäurekonzentration adipöser Pferde mit und ohne chronische Hufrehe/schlanker an chronischer Hufrehe erkrankter Pferde, die für einen kombinierten Glukose-Insulin-Test (CGIT) sowie für eine röntgenologische Untersuchung der Hufe vorgestellt wurden. Insgesamt wurden 25 adipöse Pferde und zwei schlanke Pferde mit rezidivierender Hufrehe einem CGIT unterzogen. Von den 27 Pferden, die vorgestellt wurden, waren fünf nicht Insulin-dysreguliert (adipös), 14 waren Insulin-dysreguliert und acht waren Insulin-dysreguliert mit röntgenologischen Anzeichen einer chronischen Hufrehe. Es wurden signifikante Unterschiede in den Ruhekonzentrationen der Aminosäuren (Citrullin, GABA, Methionin) zwischen gesunden und Insulin-dysregulierten an Hufrehe erkrankten Pferden festgestellt. Außerdem lagen Unterschiede in der Plasma-Konzentration von GABA zwischen Insulin-dysregulierten Pferden mit und ohne Hufrehe vor.
Insgesamt liefert dieses Projekt interessante Ansätze, weitere diagnostische Tests, insbesondere zur Detektion von Hufrehe-gefährdeten Pferde, zu entwickeln und möglicherweise auch zur Erforschung von Futterergänzungsmitteln.

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Conflicts of interest

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Declaration of independence

I hereby confirm that I have written this thesis independently. I certify that I have used only the sources and aids indicated.

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