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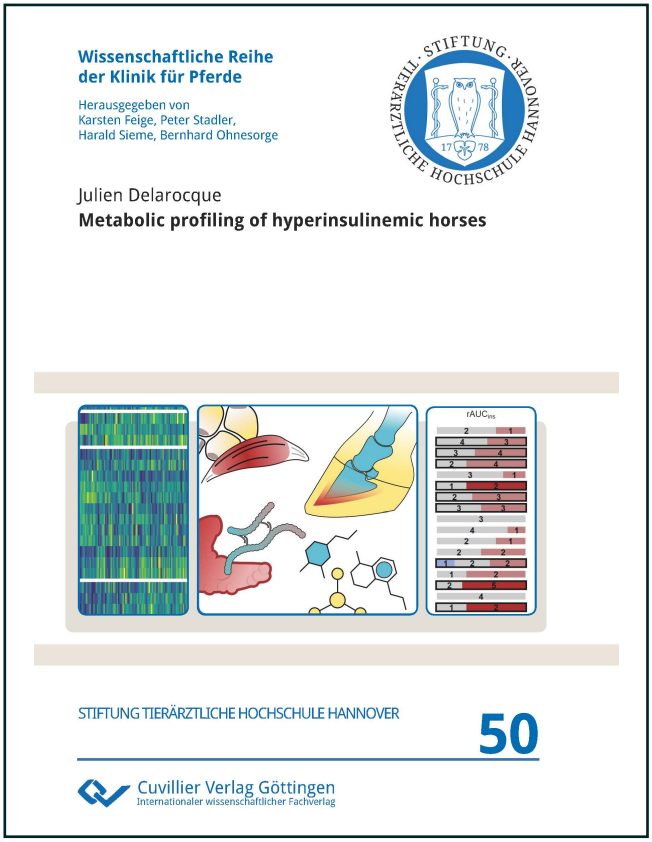
Metabolic profiling of hyperinsulinemic horses



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1. Introduction

The equine hoof consists of a horn capsule within which the distal phalanx is suspended to the heavily keratinized epidermis by outgrowths of the remarkably modified dermis called *laminae*. Thus, the weight of the animal is redirected towards the edge of the hoof wall facing the ground, while the sole is not bearing weight. Laminitis describes the failure of the attachment of the phalanx to the hoof wall and is often associated with pain and lameness [1].

The first written record of laminitis in history is commonly attributed to Xenophon (380 BC), describing a disease induced by barley surfeit [2]. Later authors also attributed laminitis to excessive grazing (grass founder) or strenuous work [2]. It is now recognized that laminitis can result from different kinds of primary diseases, such as sepsis or disruption of the gastro-intestinal barrier, or alimentary causes and excessive weight bearing, as described in early days. As a result, the research on laminitis made use of several models for laminitis induction. The widely used Obel grading system for laminitis was established using a sepsis model [3], while carbohydrate overload models became more predominant in the second half of the 20th century [2].

A possible endocrinological aetiology of laminitis was first put forward by Field and Jeffcott in 1986 [4], who linked the higher prevalence of this condition in obese ponies to their higher insulin response to an oral glucose test (OGT) compared to non-obese ponies and Standardbred horses. Retrospectively, these visionary experiments fit extraordinarily well with our current understanding of endocrinopathic laminitis. It took over twenty-five years for the term insulin dysregulation (ID), which describes the excessive insulin response to oral carbohydrates uncovered by Field and Jeffcott, to be coined [5]. Since endocrinopathic laminitis nowadays accounts for around 90% of laminitis cases [6], the underlying endocrinologic diseases have lately received considerable attention.

1.1. Equine hyperinsulinemia

Hyperinsulinemia (HI) describes an excessive insulin concentration in the blood. This can occur due to a reduced insulin clearance, as associated with insulin resistance (IR), and/or because of increased insulin secretion by the pancreatic β-cells. Pancreatic β-cell failure, as it occurs in type 2 diabetes mellitus, is very rare in horses and often associated with other diseases such as pancreatitis and endocrinologically active neoplasms [7]. Therefore, the pancreatic gland can sustain an increased insulin production for a long time, as compared to humans [8].

The term ID encompasses basal HI, IR and (transient) postprandial HI resulting from an excessive insulin response to an oral glucose stimulus. The latter was hypothesized to result from incretin stimulation, although differences in incretin concentrations in healthy and dysregulated horses could not be detected consistently [9,10]. Regardless of its underlying cause, ID results in HI at one time or another.

Since insulin secretion is part of a complex, dynamic equilibrium between energy carriers in different compartments and multidirectional hormonal control mechanisms, there is no single cut-off defining HI. The diagnostic tests for ID will be discussed later. In general, cut-offs have been established by describing the insulin response to certain test protocols in healthy and diseased cohorts, as defined by another reference test or other factors such as obesity or predisposition for laminitis [11,12]. More recently, efforts have been made to distinguish healthy and diseased animals in a less arbitrary, multivariate, clustering-based approach [13]. However, ID is neither due do congenital disorders of the metabolism (even if genetic factors can contribute to its development), nor a fundamentally irreversible state, since sufficient weight loss may normalize the insulin response of affected horses [14]. Therefore, it appears more likely that this condition can be present in different gradual intensities than that it can be described using timepoint-specific dichotomous cut-offs. As a result, the total insulin response to defined stimuli (mathematically described by the area under the insulin curve during a dynamic test) has been used as continuous measure of ID [15–19]. Nevertheless, cut-offs remain valuable in a practical setting, where it must be decided if an intervention is required or not. To add to the confusion, there are notable discrepancies between different insulin assays [20–25], so that cut-offs must be considered assay specific. All in all, the definition of HI is context dependent.

The equine metabolic syndrome (EMS) describes a range of risk factors for endocrinopathic laminitis. The term was first introduced by Johnson in 2002 [26] and its definition later clarified in two successive consensus statements [27,28]. The key feature of EMS is ID. Generalized or regional adiposity and a predisposition to weight gain are generally present and can be accompanied by secondary metabolic disorders such as hypertriglyceridemia, hypoadiponectinemia, hyperleptinemia and cardiovascular changes [28].

Another major endocrinologic disease of the horse is pituitary *pars intermedia* dysfunction (PPID). It is a neurodegenerative disorder resulting from the loss of dopaminergic inhibition of the pituitary *pars intermedia*, thus quantitatively and qualitatively altering the secretory activity of the pituitary gland. The clinical signs include hypertrichosis, muscle atrophy, polyuria and polydipsia, hyperhidrosis and abnormal fat distribution [29]. In addition, many affected horses suffer from ID, putting

them a risk for laminitis. While the mechanisms causing ID in horses with PPID are not yet fully elucidated, they might differ from the ones prevailing in horses with EMS [5]. Indeed, IR appears to play a more prominent role in PPID than in EMS, because PPID affects the peripheral glucocorticoid metabolism [30].

To summarize, PPID and EMS are the two major endocrinopathies in equids. While ID is an essential part of EMS, not all horses with PPID suffer from ID. Nevertheless, both diseases are associated with an increased risk of laminitis, which is conveyed by ID and the resulting HI. There is little epidemiological data regarding the prevalence of EMS, but the prevalence of HI was reported to be around 27% in ponies [31] and 20% in horses [32,33]. Obesity is a major risk factor for both EMS and ID and was found in approximately 20–30% of horses with seasonal variations [34,35]. In contrast, 2.9% of equids were affected by PPID in a systematic review, but this proportion increased to 21.2% in horses and ponies aged over 15 years [36].

Hyperinsulinemia can also occur in conditions promoting IR or hyperglycaemia, such as systemic infection and inflammation or gestation and hormonally active neoplasms. However, such cases will not be discussed in the present work.

1.2. Insulin-associated laminitis

Laminitis reportedly affects around 3% of equids all causes included, but the reported estimates vary greatly depending on the study population [37]. It should be stressed that this disease is painful and can require euthanasia in severe cases. The most frequent aetiology of laminitis is highlighted in the terms 'endocrinopathic laminitis' and 'insulin-associated laminitis'. The denomination 'grass founder' describes a chronic form of 'pasture-associated laminitis', which occurs in predisposed horses because of the insulin response engendered by grazing on lush pasture. The causal link between HI and laminitis was experimentally demonstrated [38–40] and is supported by the description of insulinaemia in laminitis-prone animals as compared to healthy ones [41–46].

The (human) metabolic syndrome is defined as a collection of risk factors for cardiovascular disease and type 2 diabetes mellitus [47]. While IR plays a more prominent role in humans than in horses, it is considered that HI is the initial cause of the clinical manifestations of the metabolic syndrome [48], legitimizing the term EMS. The (hypothesized) mechanisms by which HI induces IR, hypertension, dyslipidaemia, and inflammation are as manifold as the metabolic processes involving insulin. While some of these mechanisms might be transposable to equids, a mechanistic explanation of the relationship between HI and laminitis is still lacking. The previously investigated hypotheses of glucotoxicity [49] and glucose deprivation within the *laminae* [50] have been rejected. There is evidence of some forms of vascular dysfunction occurring in

laminitic horses and a model of vascular IR [51,52]; however, the potential impact on the lamellar epithelial cells remains unclear. Recently, the insulin-like growth factor-1 receptor (IGF-1R) and a hybrid insulin/ IGF-1R have been highlighted as potential binding targets for insulin in the lamellar tissue during HI [53,54]. These receptors are involved in the regulation of cell growth, adhesion, proliferation, differentiation and apoptosis [54]. The ribosomal protein S6 (RPS6) is activated by IGF-1R and regulates actin remodelling. Therefore, it could be involved in the elongation of epithelial cells, which is a major feature of lamellar histopathology in endocrinopathic laminitis [53].

1.3. Metabolic profile associated with insulin dysregulation

By definition, EMS is associated with ID, which can manifest as IR but also as permanent or transient HI. While IR is said to often occur secondarily to HI, insulin sensitive hyperinsulinemic phenotypes exist as well [10,55]. This phenotypic heterogeneity even in the most fundamental aspect of EMS explains why further metabolic dysregulations can be very variable amongst affected individuals. Likewise, EMS is often associated with obesity, but the existence of a lean EMS phenotype was also demonstrated [56,57]. As summarized in **Figure 1**, the different aspects of ID are interrelated.

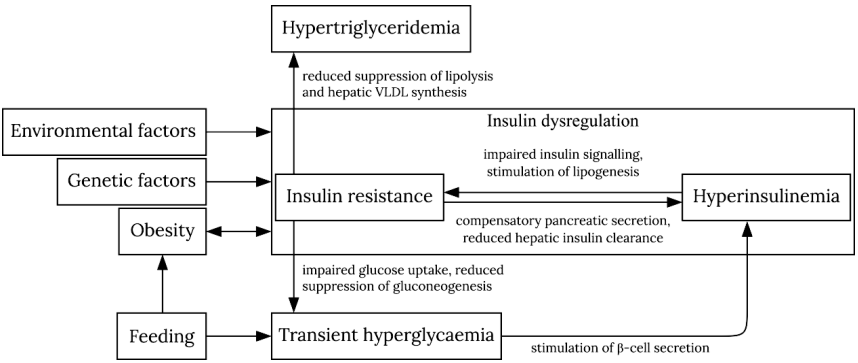

Figure 1 Graphical summary of the relationship between different aspects of EMS and their metabolic impact. VLDL: very low-density lipoprotein. Partly based on [28].

1.3.1. Previously described alterations of the metabolism

Hypertriglyceridemia is sometimes present in ID and EMS [41,56,58]. Besides triglycerides, increased non-esterified fatty acid (NEFA) [58], high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol concentrations were also reported by some authors [58,59]. Nevertheless, most lipid fractions did not consistently differ between obese IR and IS [60,61], so that dyslipidaemia might rather

be associated with further variables like the genotype, feeding, severity of obesity, etc. [61]. Hormonal differences include hyperleptinemia [58,62] and hypoadiponectinemia [63–65]. The connection between these hormones and obesity [66] nicely illustrates that it is difficult to isolate the effect of HI on the metabolism from its confounders. Such problems are also encountered when studying animals with concurrent PPID and ID, where an altered glucocorticoid metabolism might contribute to the development of peripheral IR [30].

Inflammation and oxidative stress have long been suspected to play a role in ID and laminitis [67]. However, while some mediators of inflammation such as interleukin 1 (IL-1) and tumour necrosis factor α (TNF-α) [68] appear to be associated with IR, the pathways activated by these conditions appear to be rather specific and it is difficult to support the hypothesis of a generalized (pro-)inflammatory state [69]. Nevertheless, the protracted inflammatory response to endotoxins observed in horses with EMS [70] might suggest that this condition impairs the dynamic response to certain stimuli, rather than it affects basal indicators of inflammation. In the same way, the antioxidative capacities of muscle tissue were positively correlated with the body condition score (BCS) but there was no evidence of oxidative damage [42]. Still, the available evidence regarding oxidative damage in these conditions remains contradictory [33,49,71].

1.3.2. Technical aspects

The phenotypic characterization of ID can take place on different levels. While hormone and other peptide concentrations are often determined with immunoassays [20,25,72–75], polymerase chain reaction (PCR) was mainly used to quantify gene expression [76–81]. However, the individual measurement of more broad sets of molecules can become tedious. When exploring metabolites associated with energy metabolism, high-throughput approaches relying on nuclear magnetic resonance (NMR) or mass spectrometry in combination with chromatographic techniques have become increasingly popular in both humans and livestock [82,83]. The identification of biomolecules can take place in a targeted or untargeted fashion, requiring different levels of expertise and effort (untargeted approaches being more complicated). The large range of small molecules present in an organ or organism is called metabolome and their large-scale estimation is called metabolomics. Such approaches have been employed to describe the metabolome of horses and ponies during OGT [84,85], lamellar tissue bioenergetics in an oligofructose laminitis model [86] and impact of obesity on the metabolome in horses [87]. Interesting results include an impairment of the tricarboxylic acid (TCA) cycle in insulin-dysregulated ponies [85], increase of circulating free fatty acids in obese horses [87] and signs of pro-inflammatory events during the OGT [84].

1.4. Diagnosing insulin dysregulation

Because of its implications for animal welfare, the early identification of ID is crucial. The identification of risk factors for laminitis or ID is not obvious to most horse owners [88,89] and should be communicated by the veterinarian. Testing should be considered when signs of metabolic disease become obvious (see above), significant weight gain is observed or when there is a history of laminitis [28].

Several methods for quantification of insulin have been in use in equids in recent years but did not provide perfect agreement nor a linear relationship [20–25], so that reference ranges and cut-off values should be considered assay-specific. Additionally, it is agreed that higher insulin concentrations are associated with a higher risk of laminitis. Therefore, as mentioned previously, the use of cut-off values is not necessarily the best representation of the spectrum of ID [13].

1.4.1. Basal testing

As mentioned earlier, ID encompasses basal HI, IR, and postprandial HI. Therefore, the simplest way to diagnose ID is to measure insulin in a fasted animal. An insulin concentration in blood lower than 20 µIU/ml is typically considered to rule out basal hyperinsulinemia [90]. Nevertheless, many dysregulated horses have inconspicuous basal insulin values so that the exclusion of ID based on such results is likely to be impaired by false negative errors [28].

Fasting blood glucose concentration is of little use in horses with ID, because they usually manage to maintain normoglycemia (when solely affected by ID) [12]. Hyperglycaemia is rather considered indicative of type 2 diabetes mellitus. The combination of basal glucose and insulin concentrations in ratios and proxies was found to correlate with tests for IR and might be more useful than basal glucose or insulin alone [91,92].

The use of biomarkers other than insulin and glucose (ideally in basal samples to avoid tiresome testing) has also been investigated. For example, adiponectin was reported to have 80% sensitivity and specificity to predict pasture-associated laminitis in the next three years [46] although it correlated poorly with insulin [12]. On the other hand, it remains that insulin is per definition the most sensitive indicator of ID.

1.4.2. Detecting insulin resistance

Owing to its most prominent role in human endocrinology, there are plentiful tests for the diagnosis of IR. Generally, these tests rely on the intravenous administration of glucose, insulin, or both in a predetermined dosage. The most complex of these tests is the euglycemic hyperinsulinemic clamp (EHC) [93,94], which allows to quantify several aspects of insulin and glucose dynamics using the insulin and

glucose infusion rate and is considered the gold standard. Simpler, but still laborious protocols are the frequently sampled intravenous glucose tolerance test (FSIGTT) [95] and combined glucose insulin tolerance test (CGIT) [96]. These protocols basically use glucose and insulin boluses instead of constant rate infusions to achieve similar results to the EHC. Lastly, the insulin stimulation test (IST) depends on the simple assumption that an insulin bolus should be followed by a timely reduction of the blood glucose concentration [97]. A comparatively short protocol (30 min) and a good repeatability make this test suitable for clinical use. However, the amount of information it delivers is more limited.

1.4.3. The advantages of oral testing protocols

Oral test protocols (such as the previously mentioned OGT used by Field and Jeffcott [4]) measure the insulin response to an oral glucose stimulation. The way of application and dosage of glucose (or glycaemic preparation) may vary [12,28]. While these tests are subject to greater within individual variation [12], they are more similar to naturally occurring stimuli [9,28]. The differences in the results yielded by tests of IR and oral tests arise from the fact that firstly, not all horses with ID are insulin-resistant, and secondly, glucose-related enteric mechanisms appear to play a role in ID. Consequently, oral tests are currently the recommended method for the diagnosis of ID [28].

1.5. Treating insulin dysregulation

1.5.1. Current recommendations

Since the positive correlation between obesity or weight gain and measures of IR has long been established [4], weight loss programs have been investigated early on as a possible therapy and proven rather effective [15,81,98–100]. As a result, the main recommendation for the treatment of EMS is to promote weight loss [28]. Many dietary protocols have been suggested and generally relied on the exclusion of cereal-based components and restriction of the roughage to a certain percentage of the (optimal) bodyweight [101]. It is acknowledged that some horses appear to be weight loss resistant and require even more drastic measures [14].

The concurrent promotion of exercise was often found useful [81,98,99] and some authors even reported that dietary restrictions alone were ineffective [98] – a question on which there is also no agreement in human medicine [102,103]. It could be argued that this effect relies on the endocrine activity of the musculoskeletal system which even affects adipocyte growth [104,105]. Additionally, a punctual intensive mobilization of energy storages during exercise is likely to affect their responsiveness. Nevertheless, besides methodological differences among these experiments (e.g., energy content and

source, exercise intensity and duration), there are numerous confounders which might have influenced the results (age, sex, genetic background, season, initial severity of IR to name a few). Owing to these limitations, it is also difficult to quantify the amount of weight loss required to reduce the insulin response and it remains unknown if similar results can be achieved independently of the initial bodyweight (i.e., proportionality and linearity). Lastly, most of these experiments have used measures of IR and not of ID, ignoring the enteric component of EMS [10], which might not be affected by these measures in the same way.

1.5.2. Pharmacological treatment

The pharmacological treatment of ID should not be used as a primary therapy but rather as an adjunctive one in cases refractory to weight loss [28]. Three major alternatives have been explored (1) sensitization to insulin, (2) reduction of glucose availability, and (3) increase of the metabolic rate.

The insulin-sensitizer pioglitazone is used in type 2 diabetes mellitus in humans but failed to improve IS in horses [106]. Metformin is also known as insulin-sensitizer in humans but apparently fails to reach satisfying concentrations in horses because of poor bioavailability [107]. Nevertheless, it was able to reduce estimates of IR in several trials, which was partly attributed to its effect on enterocytes [108–111]. Recently, the sodium-glucose co-transporter 2 (SGLT-2) inhibitor velagliflozin, which induces glucose loss through the renal excretion, was shown to improve HI and prevent laminitis in a preliminary trial [112]. Lastly, levothyroxine treatment helped in reducing body mass by increasing the metabolic rate and insulin sensitivity (SI) [113].

1.6. Hypotheses (H), aims (A) and objectives (O)

Insulin is responsible for the distribution of carbohydrates, fats, and further essential molecules throughout the body. Its synthesis and release are regulated by the availability of these substrates, while its effects include the synthesis of energy-carriers and proteins. Given its central role in metabolism and key position in ID, the primary hypothesis of the present thesis was that

(H1) *ID is associated with fundamental changes of the metabolome.*

To (A1.1) derive biomarkers of ID from the metabolic profile, (O1.1) the metabolic profile of horses with and without ID was compared in a basal state and during an induced hyperinsulinemia. Additionally, it was sought to (A1.2) identify pathological changes of the metabolome associated with ID by (O1.2) correlating the metabolic profile with the level of ID.

Obesity is a major risk factor for ID and weight loss is consequently the major aim of the therapy for this condition. However, previous work on the impact of obesity on

ID was primarily focused on IR and did not describe the relationship between both parameters to its full extent. As a result, it was hypothesized that

(H2) *weight loss leads to a proportional reduction of the level of ID.*

To (A2) describe the relationship between weight variations and the level of ID, (O2) the changes of the insulin response to the OGT was analysed depending on the concurrent changes in body weight.

Having addressed the relationship between ID and weight variations, it appeared important to relate these findings to the previously assessed metabolic impact of ID. As there are obese normoinsulinemic and lean insulin resistant individuals, the hypothesis that

(H3) *weight gain and aggravation of ID have a distinct impact on the metabolome*

was formulated. (A3) The concurrent description of the impact of weight gain and the fluctuations of ID on the metabolome, was achieved (O3) by correlating the metabolic profile of horses with the development of their body weight and insulin response over time during repeated OGTs.

It was then aimed to transfer the previously addressed topics to the main clinical manifestation of ID. Because insulin plays a central role in the metabolism but is also central in the pathophysiology of endocrinopathic laminitis, it was hypothesized that

(H4) *the development of laminitis can be predicted by the metabolic profile.*

Therefore, it was attempted to (A4) identify biomarkers of subsequent laminitis development by (O4) comparing the metabolome of laminitis-resistant and laminitis-prone ponies.

Since endocrinopathic laminitis is mostly associated with long-standing metabolic dysregulation and/or obesity, it was finally investigated whether

(H5) *the subsequent development of laminitis is primed by pre-existent metabolic changes.*

To (A5) identify metabolic differences associated with subsequent laminitis, (O5) the basal metabolome and metabolic response to a high-sugar diet of laminitis-resistant and laminitis-prone ponies were compared.