Endocrine response to illness in sick horses

Presence of critically-illness-related corticosteroid insufficiency or pancreatic insufficiency in horses is related to poor survival

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Cover: “The reason for it all: increasing patient survival increases owner happiness, and happiness is one of the greatest measures of quality of life”
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Endocrine response to illness in sick horses. Presence of critically-illness-related corticosteroid insufficiency or pancreatic insufficiency in horses is related to poor survival

Abstract

Endocrinological responses are under tight physiological control in healthy animals, with homeostatic adaptions made for diet, exercise and illness. Transient adrenal dysfunction with inadequate cortisol production, known as relative adrenal insufficiency (RAI), is a major component of critical illness–related corticosteroid insufficiency (CIRCI), which involves dysfunction of the entire HPA axis. Recognition and treatment of CIRCI improves human survival rates. Whereas a low-dose ACTH stimulation test appears to be the most sensitive method of diagnosing CIRCI in people, published doses for this test in horses were considerably higher, and the lowest dose of ACTH that will maximally stimulate the adrenal glands of horses was unknown. Critical illness is also associated with peripheral tissue insulin resistance and hyperglycaemia in many species, but insulin and glucose dynamics in spontaneously sick adult horses remains to be investigated.

The overall aim was to document changes in cortisol, ACTH, insulin and glucose in sick horses; determine if adrenal or pancreatic dysfunction occurred, and which parameters were correlated with non-survival. A secondary aim was to validate the low-dose ACTH stimulation test in healthy adult horses and neonatal foals and then determine if CIRCI could be identified in sick horses.

A dose of synthetic ACTH of 0.1 µg/kg in healthy adult horses and 0.25 µg/kg in neonatal foals resulted in maximal adrenal stimulation. In sick horses, cortisol, ACTH and ACTH/cortisol ratios were higher in non-survivors at admission. ACTH/cortisol ratios were higher in horses with systemic inflammatory response syndrome (SIRS) and those with an ischemic gastrointestinal lesion, suggesting adrenal dysfunction. Although horses with ischemic lesions had higher basal cortisol, they failed to respond to ACTH stimulation, further indicating adrenal dysfunction. RAI/CIRCI was diagnosed in 21% of sick horses and is associated with a poorer prognosis. Horses presenting with hyperglycaemia and corresponding relative hypoinsulinemia (suggestive of endocrine pancreas dysfunction) had a worse prognosis.

Keywords: endocrinology, equine, inflammation, CIRCI, adrenal, cortisol, adrenocorticotropic hormone, ACTH stimulation, SIRS, insulin dysregulation

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Endokrin respons på sjukdom hos häst: Förekomst av sjukdomsrelaterad sekundär kortisolsvikt eller pankreasinsufficiens är associerat med sämre överlevnad.

Abstract

Endokrina responser är strikt reglerade hos friska djur och anpassas efter diet, fysiskt arbete och sjukdom. Övergående binjuredysfunktion ger otillräcklig kortisolproduktion, sk ”relative adrenal insufficiency”, RAI. Detta spelar en viktig roll i sjukdomsrelaterad sekundär kortisolsvikt (”critical illness-related corticosteroid insufficiency”, CIRCI) där man har dysfunktion av hela HPA axeln. Identifiering och behandling av sekundär kortisolsvikt förbättrar överlevnaden inom humanvården. Lågdos ACTH-stimuleringstest förefaller vara det känsligaste testet för diagnos, men publicerade doser för att utföra provtagning på häst har varit betydligt högre än vad som används på humansidan. Den lägsta dos ACTH som krävs för maximal stimulering av hästens binjure har hittills varit okänd. Allvarlig sjukdom är också associerad med perifer insulinresistens och hyperglykemi på flera djurslag, men mer information om insulin- och glukosmetabolismen hos sjuka hästar behövs.

Målet med studien var att dokumentera förändringar i kortisol, ACTH, insulin och glukos hos sjuka hästar, att fastställa eventuell förekomst av binjure- eller pankreasinsufficiens, samt att undersöka vilka parametrar som kan korreleras till sämre prognos för överlevnad. Målet var också att validera ett lågdos ACTH-stimuleringstest för användning på vuxna hästar och neonatala föl, och att fastställa om CIRCI kunde detekteras hos sjuka hästar.

Syntetiskt ACTH doserat till 0,1µg/kg åt friska hästar och 0,25µg/kg åt friska neonatala föl resulterade i maximal binjurestimulering. Hos sjuka hästar som provtogs vid ankomst till klinik var kortisol, ACTH, och ACTH/kortisol kvoten högre hos de hästar som inte överlevde. ACTH/kortisol kvoten var högre hos hästar med systemiskt inflammationsrespons syndrom (SIRS) och hos hästar med ischemiska tarmlidanden, vilket indikerar binjuredysfunktion. Hästar med ischemiska skador hade också högre basala kortisolhalter, men svarade dåligt på ACTH-stimulering vilket ytterligare indikerar binjuredysfunktion. RAI/CIRCI diagnosticerades hos 21 % av de sjuka hästarna och var associerat med sämre prognos. Hästar som kom in med hyperglykemi och samtidig relativ hypoinsulinemi (överensstämmande med dysfunktion av endokrina pankreas) hade en sämre prognos för överlevnad.

Keywords: endokrinologi, häst, inflammation, CIRCI, adrenal, kortisol, ACTH, ACTH-stimuleringstest, SIRS, insulin dysfunktion

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Preface

The basis of this research originally stemmed from my interests in emergency and critical care medicine and endocrinology. Although animals are often used as research models for human disease or pharmaceutical testing, I like to find pharmaceuticals or diagnostic tests that have been proven safe and efficacious in human medicine, and then determine appropriate dosing in horses. Ellen Behrend and Linda Martin were two of my most influential mentors when I was a new faculty member at Auburn University, and this work in horses was originally adapted from work undertaken by them in dogs. To Joe Mayhew and Anna Kendell who suggested I utilise this body of work as part of the requirements for a PhD. To John Pringle and Pia Anderson for mentoring me at SLU and Ulva Sjunnesson and Björn Ekesten for accepting me into the SLU PhD program. Finally to my nearly 4 year-old daughter Laura Margaret Stewart for being so patient and happily enjoying many repeated screenings of four Disney DVDs during the weekends I spend writing the final manuscripts and thesis. I owe her a holiday at the beach.

Dedication

To the patients we try to save and the students we try to teach, who in turn teach us something new every day

Chance favours the prepared mind.
Louis Pasteur

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Stewart, A.J.*, Behrend, E.N., Wright, J.C., Martin, L.G., Kemppainen. R.J., Busch, K.A., Hanson, R.R. (2011). Validation of a low-dose ACTH stimulation test in normal adult horses. *Journal of the American Veterinary Medical Association*. 239(2), 834-841.

II Stewart, A.J.*, Wright, J.C., Behrend, E.N., Martin, L.G., Kemppainen, R.J., Busch, K.A. (2013). Validation of a low-dose ACTH stimulation test in healthy neonatal foals. *Journal of the American Veterinary Medical Association*. 243(3), 399-405.

III Bertin, F.R., Ruffin-Taylor, D., Stewart, A.J.* (2018). Insulin dysregulation in horses with systemic inflammatory response syndrome. Journal of Veterinary Internal Medicine. 32(4):1420-1427.

IV Stewart, A.J.*, Hackett, E., Bertin, F. R., Towns, T.J. Cortisol and ACTH concentrations in horses with systemic inflammatory response syndrome. Manuscript

V Stewart, A.J.*, Hackett, E., Bertin, F.R., Towns, T.J. Identification of Adrenal Dysfunction in Critically Ill Adult Horses. Manuscript

*Corresponding author

The contribution of Allison J Stewart to the papers included in this thesis was as follows:*

I 90%

II 90%

III 85%

IV 85%

V 85%

Related publications not included in the thesis

Bertin, F.R., Yuen, K.Y[†], Hinrichsen, S.[†], Horn, R¥.¥., Stewart, A.J.* Effects of sample processing on the stability of ACTH stability in horses with normal and elevated ACTH concentrations. *Journal of Veterinary Internal Medicine.* Manuscript in review

Stewart AJ* (2017) Testing and Monitoring for Pars Intermedia Dysfunction (PPID). *The Veterinarian*. December: 20-21

Stewart AJ* (2015) Review Article: Pituitary pars intermedia dysfunction. *Australian Equine Veterinarian*. 34 (4):59-67 *Winner Australian Equine Veterinarian's John Bourke Literary Prize*

Dembek, K.A.[§], Hurcombe, S.D., Stewart, A.J., Barr BS, MacGillivray KC, Toribio RE. (2016) Association of aldosterone and arginine vasopressin concentrations and markers of hypoperfusion in neonatal foals. *Equine Veterinary Journal*. 48(2):244-257 doi/10.1111/evj.12393/pdf

Stewart, A.J.* (2013) Update on the diagnosis of equine endocrine disorders. *Equine Veterinary Practitioner.* 27(3):29-39

Funk, R.A.[¥], Wooldridge, A.A., Stewart, A.J., Behrend, E.N., Zhong, Q., Kempainen, R.J., Johnson, A.K. Seasonal changes in the combined glucose insulin tolerance test in normal aged horses. (2012) *Journal of Veterinary Internal Medicine*. 26:1035-1041.

Schreiber, C.M.[†], Stewart, A.J.*, Behrend, E.N., Wright, J., Kwessi, E.[§], Kemppainen, R., Busch, K.A. (2012) Seasonal variation in diagnostic tests

for pituitary pars intermedia dysfunction in normal aged geldings. *Journal of the American Veterinary Medical Association.* 241(2):241-248.

Funk, R.A.[¥], Stewart, A.J.*, Wooldridge, A.A., Kwessi, E.[§], Kemppainen, R.J., Behrend, E.N., Johnson, A.K. (2011) Seasonal changes in plasma alpha melanocyte stimulating hormone and adrenocorticotropic hormone in response to thyroid releasing hormone administration in normal aged horses. *Journal of Veterinary Internal Medicine*. 25:579-585.

Wong, D.M., Vo, D.T., Alcott, C.J., Stewart, A.J., Peterson, A.D., Sponseller, B.A., Hsu, W.H. Adrenocoricotropic hormone stimulation tests in healthy foals from birth to 12 weeks of age. *Canadian Journal of veterinary Research*. 2009: 73(1):65-72.

Stewart, A.J.* (2006). Relatív mellékvese-elégtelenség. Előfordul-e súlyosan beteg lovakban? (Relative adrenal insufficiency. Does it exist in critically ill horses?) *Magyar allatorvosok lapja (Hungarian Veterinary Journal*). 128(12), 707-711.

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Abbreviations

ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
AVP	Arginine vasopressin
CBC	Complete blood count
CIRCI	Critical illness–related corticosteroid insufficiency
CRH	Corticotrophin-releasing hormone
EMS	Equine metabolic syndrome
HPAA	Hypothalamic-pituitary-adrenal gland axis
IL1-β	Interleukin 1-β
mRNA	Messenger ribonucleic acid
LC-MS	Liquid–chromatography mass spectrometry
LPS	Lipopolysaccharide
MODS	Multiple organ dysfunction
OR	Odds ratio
PPID	Pituitary pars intermedia dysfunction
RAI	Relative adrenal insufficiency
ROC	Receiver operating characteristic
SCCM	Society of Critical Care Medicine
SD	Standard deviation
SIRS	Systemic inflammatory response syndrome
TNF-α	Tumour necrosis factor-α

1 Summary and Introduction

1.1 Summary

Endocrinological responses are under tight physiological control in the healthy animal, with homeostatic adaptions made for diet, exercise and illness. The "stress response" involves recognition of a stressful situation by the amygdala, release of corticotrophin releasing hormone (CRH) from the hypothalamus which acts on the pituitary gland to produce adrenocorticotrophin (ACTH), and adrenal gland release of cortisol. Cortisol helps to maintain the bodies' fluid balance, blood pressure, increases blood glucose and suppresses the immune system. Physiologic balance is crucial, with excessive or inadequate cortisol being detrimental. With illness, it is expected that CRH, ACTH, cortisol and glucose would increase; however how the exact concentrations of respective hormones corresponds to a particular degree of illness is unknown. With prolonged critical illness and organ dysfunction, regulatory hormone concentrations may be inadequate to compensate for the systemic effects of illness.

Systemic inflammatory response syndrome (SIRS) occurs due to increased release of inflammatory mediators (cytokines, lipid mediators, proteases, vasoactive peptides) and/or decreased production of anti-inflammatory substances, and can lead to shock, multiple organ damage and eventually death. Concentrations of inflammatory mediators such as tumour necrosis factor-α, interleukin-1, and interleukin-6 are increased. These suppress secretion of CRH and ACTH, cause ACTH resistance, and alter cortisol secretion and metabolism. Transient adrenal dysfunction and inadequate cortisol production is known as relative adrenal insufficiency (RAI) and is a major component of critical illness–related corticosteroid insufficiency (CIRCI), which involves dysfunction of the entire HPA axis, including tissue corticosteroid resistance. Approximately half of human high-risk critically ill patients have CIRCI. This condition can result in a protracted and excessive inflammatory response and is associated with increased morbidity and

mortality rates. Recognition and treatment of CIRCI dramatically improves human survival rates.

There are descriptions of CIRCI in dogs, cats and neonatal foals. It is not known if CRICI exists in critically ill adult horses. While it has been shown that high admission baseline cortisol concentrations are associated with non-survival in equine colic cases, delta cortisol, ACTH/cortisol ratios, and hormonal dysfunction throughout hospitalisation have never been assessed in horses. Also, extensive adrenal haemorrhage and adrenocortical necrosis are common necropsy findings of critically ill horses.

Diagnosis of CIRCI involves identification of a high ACTH-to-cortisol ratio or low delta cortisol (difference between baseline and ACTH-stimulated cortisol concentrations).Prior to this work the lowest published dose used for ACTH stimulation tests in adult horses was was 250 µg/horse or 0.5 µg/kg. This choice of dose is probably based on the tradition from human medicine, where supraphysiologic doses of ACTH (250 µg) are administered to diagnose Addison's disease (absolute adrenal insufficiency) in human patients. The lowest dose of ACTH that will maximally stimulate the adrenal glands of horses is unknown, but it seems that use of a low-dose (1-2 µg) ACTH stimulation test appears to be more sensitive at diagnosing CIRCI.

Peripheral tissue insulin resistance and hyperglycaemia occur secondary to sepsis or severe trauma in many species, and elevated blood glucose concentration has been associated with non-survival. Regulation of blood glucose concentration by intensive insulin therapy has improved survival in some human studies, but not in others. While experimental lipopolysaccharide infusions have been shown to induce peripheral tissue insulin resistance in horses, the insulin and glucose dynamics in spontaneously sick adult horses has yet to be investigated.

Since both RAI and CIRCI are associated with decreased survival in sick horses, the overall aim of this research was to develop methods for and testing adrenal function in foals and adult horses, in addition to the pituitary, adrenal and pancreatic responses to illness in hospitalized adult horses.

1.2 Introduction

1.2.1 HPA axis

Stress from fear and excitement in healthy animals and inappetence, dehydration, trauma, pain, and the effects of inflammation such as hypotension, hypoxemia or fever in sick animals activates the HPA axis. The parvocellular neurons located in the paraventricular nucleus of the hypothalamus synthesize CRH, which is released into the portal system of the median eminence by neurosecretory nerve terminals and then binds to the CRH-1 receptor on the corticotropic of the pituitary gland (Belda *et al.*, 2015; Keller-Wood, 2015). Factors shown to influence CRH secretion include inputs from the

suprachiasmatic nucleus of the hypothalamus and the pineal gland which result in HPA activation in a circadian fashion with highest concentrations of cortisol occurring in the morning and lowest in the evening (Belda *et al.*, 2015; Keller-Wood, 2015). In horses, ACTH is released in peaks occurring approximately 10 times per hour (Alexander *et al.*, 1996). Seasonal and geographic variations in ACTH concentration also occur in horses, with higher values observed in the autumn, requiring seasonal and geographic specific reference ranges (Cordero *et al.*, 2012; Schreiber *et al.*, 2012; Funk *et al.*, 2011; Beech *et al.*, 2009; Donaldson *et al.*, 2005). In illness CRH secretion is also influenced by lipopolysaccharide, cytokines, brain-derived neurotropic factor, neurosteroids, γ-aminobutyric acid, glutamate, nitric oxide as well as via the negative feedback effect of glucocorticoids (Dembek & Toribio; Belda *et al.*, 2015; Keller-Wood, 2015). Corticotrophin-releasing hormone and arginine vasopressin stimulate release of ACTH from corticotropes in the pituitary *pars distalis*, and this is moderated by lipopolysaccharide, cytokines, nitric oxide and glucocorticoids during illness and trauma (Beishuizen & Thijs, 2003; Turnbull & Rivier, 1999). The relative influence of arginine vasopressin and CRH on ACTH secretion is species specific, but in health CRH is the most important pituitary secretagogue in horses based on an *in vitro* study (Evans *et al.*, 1993). However in an endotoxin challenge model vasopressin was a greater stimulant of ACTH release than CRH (Alexander & Irvine, 2002). Other stimulators of ACTH secretion include oxytocin, angiotensin II and β-adrenergic agents. B-endorphins, encephalin and somatostatin decrease ACTH release (Belda *et al.*, 2015; Keller-Wood, 2015).. In humans ACTH has a half-life of 10-15 minutes (Guyton & Hall, 1996). The pituitary gland is connected to the hypothalamus by the infundibular stalk. The anterior lobe (adenohypophysis) in horses wraps around the other two lobes, and is divided into the *pars distalis* and *pars tuberalis.* Cells in the *pars distalis* include corticotropes, somatotropes, gonadotropes, thyrotropes and lactotropes. The corticotropes synthesize and secrete ACTH, which is the main proopiomelanocortin (POMC) derived peptide (Guyton & Hall, 1996). The *pars intermedia* contains mainly melanotropes and becomes hypertrophied with pituitary pars intermedia dysfunction (PPID) due to loss of dopaminergic inhibition *(McFarlane & Cribb, 2005)* In the melanotropes POMC is mainly cleaved into α-melanocyte stimulating hormone, in addition to ACTH, β-endorphin and corticotropin-like intermediate peptide. The pars nervosa stores and releases arginine vasopressin and oxytocin that is produced in the hypothalamus (Guyton & Hall, 1996).

The adrenal glands are composed of the medulla which consist of catecholamine secreting chromaffin cells. In response to sympathetic stimulation there is secretion of adrenaline, nor-adrenaline and dopamine (Guyton & Hall, 1996). The adrenal cortex is composed of 3 zones. The innermost *zona reticularis* which produces sex steroids such as androgens, the *zona glomerulosa* which secretes the mineralocorticoid aldosterone in response to angiotensin-II as part of the renin-angiotensin-aldosterone system. Aldosterone secretion is also increased in response to changes in extracellular fluid osmolality and

hyperkalemia. The *zona fasciculata* is the middle layer of the adrenal cortex and secretes glucocorticoids (cortisol), which decrease CRH, vasopressin and ACTH secretion by negative feedback (Guyton & Hall, 1996). Cortisol increases gluconeogenesis, decreases glucose utilization, increases serum glucose and inhibits the effects of insulin. The HPA axis regulates homeostatic systems that modulate metabolism, thirst, electrolyte balance, blood pressure, pain, immunity and sexual behavior. It is also responsible for the stress response to external and internal factors that ultimately ensures maintenance of organ function and individual survival. Cortisol has a negative feedback on the hypothalamus and pituitary gland to inhibit CRH and ACTH release (Guyton & Hall, 1996).

1.2.2 Cortisol synthesis

The adrenal cortex has a limited storage of steroids. Steroidogenesis is regulated by ACTH in an acute rapid process as well as in a long-term chronic process. The G-protein-coupled melanocortin type 2 receptor binds ACTH to stimulate the transcription and translation of steroidogenic acute regulatory protein (StAR) (Miller & Auchus, 2011). Cholesterol is transferred from the outer to the inner mitochondrial membrane by StAR, where it is converted into pregnenalone which is a rate limiting step (Miller & Auchus, 2011). ACTH increases circulatory cholesterol uptake into the adrenal cortex, in addition to increasing transcription of genes encoding steroidogenic enzymes such as hydroxysteroid dehydrogenases and cytochrome P450 enzymes. Adrenocortical hyperplasia and hypertrophy is promoted by ACTH stimulated synthesis of insulin-like growth factor II, fibroblast growth factor and epidermal growth factor (Miller & Auchus, 2011). Once pregnenalone is transported to the endoplasmic reticulum it is converted to progesterone which in the zona glomerulosa is converted to aldosterone, while in the *zona fasciculata* progesterone is converted to 17α – hydroxyprogesterone and then cortisol. Pregnenalone can also be converted to 17α–pregnenolone to 17α–progesterone and cortisol (Guyton & Hall, 1996).

1.2.3 Cortisol availability

A single point in time cortisol concentration may not be reflective of the overall severity of illness as cortisol metabolism and function may also be altered during critical illness. Cortisol in plasma is 90% bound to an alpha-globulin called corticosteroid-binding globulin (CBG or transcortin) and albumin. Only 5-10% is in the free physiologically active form in healthy individuals. Elastase cleaves CBG, decreasing its affinity for cortisol and increasing the free cortisol fraction at sites of inflammation (Pemberton *et al.*, 1988). Also due to saturation of binding proteins any increases in cortisol greater than approximately 25 μg/dL in humans resulting in increases in the free fraction of cortisol. As hyperproteinemia is common in critically ill

patients this also will raise the unbound and biologically active form of cortisol.

Reduced cortisol degradation due to reduced expression and activity of cortisol metabolizing enzymes; (Boonen *et al.*, 2013) renal dysfunction prolonging the half-life of circulating cortisol, and diminished concentrations of albumin and cortisol binding globin lead to increases in physiologically active free-cortisol, (Boonen *et al.*, 2013; Hart *et al.*, 2011; Hamrahian *et al.*, 2004) influence serum free and total cortisol concentrations and the effectiveness of glucocorticoid receptor binding. In normal humans the half-life of cortisol is 60-90 minutes. In critically ill humans total serum cortisol was found to increase 2-3 times higher than that of healthy volunteers, while free cortisol concentrations increased 7-10 times (Khan *et al.*, 2004). Although measurement of total cortisol may underappreciate the HPA response to illness, measurement of free cortisol is technically difficult and not routinely available, and showed no advantage to serum total cortisol in the prediction of disease severity or outcome in septic foals (Hart *et al.*, 2011). During stressful states such as illness from inflammation, infection or trauma there is an increase in CRH and ACTH secretion, overriding cortisol negative feedback effects resulting in further elevations of cortisol ideally in proportion to the severity of illness (Guyton & Hall, 1996).

1.2.4 Cortisol function

Glucocorticoids have important roles in metabolism, the immune system and the cardiovascular system. Stress, cytokine release, tissue damage, hypoglycaemia, hypotension and hypoxia stimulate the secretion of cortisol (Guyton & Hall, 1996). Cortisol stimulates muscle catabolism to liberate proteins and promotes gluconeogenesis to produce glucose from amino acids. Cortisol also increases the lipolytic effect of catecholamines (Guyton & Hall, 1996). Cortisol increases myocardial and vascular smooth muscle responsiveness to epinephrine and norepinephrine and enhances water excretion. In the absence of glucocorticoids these processes are impaired. Glucocorticoids have an important function in moderating the inflammatory cascade and are used commonly at pharmacological doses for their anti-inflammatory and immunosuppressive effects. They increase peripheral neutrophil counts and reduce peripheral lymphocyte, monocyte and eosinophil counts resulting in the classic "stress leukogram" (Guyton & Hall, 1996).

1.2.5 HPA axis in horses

Several studies have investigated the HPA axis in normal horses. Circadian rhythm exists in horses, with cortisol peaks between 0600 and 0900 hr and troughs between 1900 and 2300 hr, (Irvine & Alexander, 1994; Toutain *et al.*, 1988; Eiler *et al.*, 1979a; Hoffsis *et al.*, 1970) but this rhythm is obliterated by minor perturbation (Irvine & Alexander, 1994). Cortisol concentrations have

been measured in exercising horses, (Marc *et al.*, 2000; Golland *et al.*, 1999; Jimenez *et al.*, 1998; Lassourd *et al.*, 1996; Ralston *et al.*, 1988; Baker *et al.*, 1982; Snow & Rose, 1981; James *et al.*, 1970), sick pregnant mares, (Santschi *et al.*, 1991a) equine colic patients, (Mair *et al.*, 2014; Hinchcliff *et al.*, 2005; Hoffsis & Murdick, 1970) and normal, (Stewart *et al.*, 2013; Wong *et al.*, 2009b; Rossdale *et al.*, 1973) premature (Silver *et al.*, 1984) and septic foals (Dembek *et al.*, 2013; Wong *et al.*, 2009a; Hart *et al.*, 2011; Hurcombe *et al.*, 2008; Gold *et al.*, 2007).

Horses are distinct from other domestic species in that they have a short period of pre-partum foetal endocrinological maturity (Silver, 1992). Although plasma ACTH concentrations normally increase over the last 10 days of gestation, foetal serum cortisol concentration remains low until 4-5 days before birth, then rises exponentially in the last 24-48 hrs prior to birth (Cudd *et al.*, 1995; Silver & Fowden, 1994). Cortisol concentrations in normal, term foals continue to rise immediately after birth, peaking within the first hour of life, (Silver & Fowden, 1994) and then decline to previous levels by 4-12 hrs after birth (Rossdale *et al.*, 1982). The rise in foetal cortisol concentration is associated with a rise in foetal ACTH concentration, (Fowden & Silver, 1995) suggesting a mature HPA axis at birth in normal term foals (Rossdale *et al.*, 1982).

1.2.6 Assessment of adrenocortical function

The function of the adrenal gland is assessed by performing an ACTH stimulation test, in which exogenous ACTH is injected intravenously or intramuscularly and cortisol concentrations are measured before and after injection. A normal adrenocortical response involves an increase in cortisol concentration, while a state of absolute adrenal insufficiency is diagnosed by low basal cortisol concentrations and failure to elevate cortisol concentration post ACTH administration.

1.2.7 Adrenal insufficiency

Absolute hypoadrenocorticism in humans is known as Addison's disease, and may be primary due to failure of glucocorticoid (and mineralocorticoid) production from adrenocortical dysfunction, or secondary from failure of ACTH production due to pituitary dysfunction. Addison's disease is not uncommon in young to middle aged dogs, but has not been diagnosed in horses.

1.2.8 Adrenal insufficiency and exhaustion in adult horses

Transient adrenal insufficiency has been reported in a horse associated with long-term anabolic steroid administration, (Dowling *et al.*, 1993) and in a

neonatal foal secondary to sepsis (Couetil & Hoffman, 1998). In contrast to dogs and humans, iatrogenic hypoadrenocorticism secondary to glucocorticoid therapy is uncommon in horses. Although not well described in the literature there are anecdotal reports of suspected adrenal exhaustion in over-trained racehorses. One study in racing thoroughbreds found that although cortisol and ACTH concentrations decreased as the fitness of the horses increased, the adrenal gland response to exogenous ACTH was not altered by training (Wilson *et al.*, 1991). Furthermore, low serum cortisol concentrations were not found in stressed racehorses with a history of poor performance (Baker *et al.*, 1982). Therefore evidence of adrenal insufficiency in racehorses is lacking.

1.2.9 History of cortisol use in critical care patients insufficiency

Corticosteroid use in sepsis was first published in 1951, where there was a documented reduction in complications post streptococcal infection in a cohort of patients with the use of cortisone (Hahn *et al.*, 1951). There were other studies showing beneficial results in patients with typhoid and sepsis. However in studies using 30 mg/kg of methylprednisolone in severe sepsis, comparative studies either showed no benefit or increases in mortality (Rai *et al.*, 2004). Thereafter the use of glucocorticoids was discouraged. Nonetheless, after studies in patients with meningitis and sepsis showed beneficial results with the use of corticosteroids, there has been renewed interest in their use for specific conditions at low dosages (Annane *et al.*, 2002; de Gans & van de Beek, 2002).

1.2.10 Activation and dysfunction of the endocrine system in critically ill patients

Sepsis, septic shock, trauma and the systemic inflammatory response syndrome (SIRS) can have dramatic effects on endocrine function (Jarek *et al.*, 1993). Stress from many sources, including illness, dehydration, pain, fever, hypotension, inappetance, hypoxemia and tissue trauma activates the HPA axis with sustained release of CRH from the hypothalamus, ACTH from the pituitary and cortisol from the adrenal cortex (Jimenez *et al.*, 1998; Lassourd *et al.*, 1996; Messer *et al.*, 1995; Furr *et al.*, 1992; Santschi *et al.*, 1991a; Hoffsis & Murdick, 1970). Increases in circulating glucocorticoid concentrations are an essential component of the stress response, which is necessary for homeostasis during critical illness. Cortisol concentrations have been shown to increase after a variety of stressors, and the response is typically proportional to the magnitude of the injury or disease process (Munck *et al.*, 1984). Inflammatory cytokines such as TNF-α, interleukin1-β, and lipopolysaccharide increase AVP, CRH and ACTH mRNA expression and secretion (Gadek-Michalska *et al.*, 2013). Experimental lipopolysaccharide administration results in increases in AVP, CRH, ACTH and cortisol concentrations (Gadek-Michalska *et al.*, 2013; Beishuizen & Thijs, 2003; Dadoun *et al.*, 1998). The acute adaptive

response of the HPA axis to stress is to release hormones from storage and upregulate hormone synthesis. However, this can ultimately lead to consumption of precursors, or maximization of rate-limiting enzymatic reactions and homeostatic mechanisms becoming incapable of keeping up with demands. The consequence of inflammatory mediators and microorganism products can result in inappropriately low concentrations of vasopressin, reduced adrenal responsiveness to ACTH, euthyroid sick syndrome, hyperleptinemia, hyperglycaemia and insulin resistance (Gheorghiță *et al.*, 2015). Endocrine organs can also be victims of the effects of SIRS and sepsis, with multiple organ dysfunction (MODS) causing irreversible damage to the pancreas, pituitary and adrenal glands as well as other organs.

1.2.11 Relative adrenal insufficiency (RAI) and critical illness–related corticosteroid insufficiency (CIRCI)

Although plasma cortisol and ACTH concentrations are expected to be high during acute illness, a loss of correlation between ACTH and plasma cortisol concentrations has been observed in critically ill human patients, (Moran *et al.*, 1994; Sibbald *et al.*, 1977) and ACTH concentration as well as basal and ACTH-stimulated cortisol concentrations are often astonishingly low (Soliman *et al.*, 2004). Inflammatory mediators also cause ACTH resistance and alter cortisol secretion and metabolism (Cooper & Stewart, 2003; Beishuizen & Thijs, 2001; Zaloga & Marik, 2001). A prolonged inflammatory condition can lead to a blunting of HPA activation leading to transient adrenal insufficiency with inadequate production of cortisol to meet the demands of the severity of illness which is termed relative adrenal insufficiency (RAI). This can progress to absolute adrenal insufficiency which may be transient or permanent. Adequate adrenocortical function is essential for patient survival, but there is still debate on what constitutes an appropriate HPA response to critical illness (Annane *et al.*, 2018). Although absolute hypoadrenocorticism is rare in human ICU patients, RAI is increasingly being identified (Beishuizen & Thijs, 2001; Jurney *et al.*, 1987). In human ICU's, RAI is commonly caused by sepsis and SIRS, (Zaloga & Marik, 2001) with inflammatory mediators released in response to illness suppressing the release of CRH and ACTH leading to impairment of cortisol synthesis. Severe thrombosis and haemorrhage of the adrenal glands can lead to RAI (Cooper & Stewart, 2003; Zaloga & Marik, 2001). Haemorrhagic adrenal necrosis as a complication of severe sepsis is called Waterhouse-Friderichsen Syndrome and can result in severe adrenal insufficiency (Bornstein, 2009). Septic human patients have developed multiple endocrine failure with decreased production of ACTH, arginine vasopressin, thyroid hormones and growth hormone as the result of pituitary necrosis (Prigent *et al.*, 2004). The encompassing term of critical illness–related corticosteroid insufficiency (CIRCI) is characterized by dysfunction of any part of the HPA axis, altered cortisol metabolism, and tissue resistance to glucocorticoids. Dysregulation of the HPA axis involves crosstalk between the

CRH -ACTH- vasopressin pathways, and the immune and autonomic nervous systems. Limited recent studies have shown marked reduction in plasma clearance of cortisol during critical illness, due to suppressed expression and activity of cortisol-metabolizing enzymes in the kidneys and liver. Despite elevated cortisol concentrations during critical illness insufficient glucocorticoid alpha-mediated anti-inflammatory activity resulting in tissue resistance to glucocorticoids has been demonstrated (Annane *et al.*, 2018).Although some authors recommend replacing the older term of RAI with CIRCI, RAI is really a distinct subset of CIRCI involving adrenal dysfunction. As adrenal function is the most widely tested component of CIRCI the term RAI/CIRCI has been adopted when CIRCI is diagnosed due to adrenal dysfunction (Dembek & Toribio, 2018) Although tissue glucocorticoid resistance has been documented it is difficult rarely measured in clinical patients. Compared with hypothalamic or pituitary dysfunction, adrenocortical failure is the most common clinically observed condition affecting the HPA axis in people and foals (Annane *et al.*, 2018; Dembek & Toribio, 2018). HPA axis dysregulation results in a diagnosis of RAI or CIRCI in ~30-45% of high risk critically ill humans, (Annane *et al.*, 2002; Beishuizen & Thijs, 2001; Rivers *et al.*, 2001; Soni *et al.*, 1995; Moran *et al.*, 1994; Sibbald *et al.*, 1977) and is associated with increased morbidity and mortality.(Soliman *et al.*, 2004; Rivers *et al.*, 2001; Bollart *et al.*, 1998; Sibbald *et al.*, 1977) Recognition and treatment of CIRCI dramatically improves survival (Soliman *et al.*, 2004; Rivers *et al.*, 2001; Bollart *et al.*, 1998; Sibbald *et al.*, 1977)

1.2.12 RAI/CIRCI in horses – background to this work

It is likely that CIRCI occurs in critically ill horses as in other species(Stewart, 2006). Couetil reported transient RAI in a septic neonatal foal that was successfully treated with prednisone.(Couetil & Hoffman, 1998) . However prednisone has subsequently been shown to have very poor bioavailability in horses.(Peroni *et al.*, 2002)) In the 1970's and 80's Rossdale, Silver, Fouden and Ousey studied prematurely and the effects of the HPA axis on the readiness for birth. They studied prematurely induced foals and found that they had low serum cortisol concentrations at birth (4-5 times lower than term Thoroughbred and term induced pony foals). These prematurely induced foals had no significant rise in cortisol concentration over the next 2 hours as seen in normal foals.(Rossdale *et al.*, 1982; Nathanielsz *et al.*, 1975) They also had an absolute neutropenia, low neutrophil to lymphocyte ratio (N/L) and a poor prognosis for survival (Rossdale *et al.*, 1982). To diagnose RAI in these prematurely induced foals Rossdale used a high-dose (0.125 mg/foal) ACTH stimulation test and advocated this as a method of assessing maturity (Rossdale *et al.*, 1982). He found that serum cortisol concentration in the term foals increased in response to ACTH, while no response was seen in the premature foals. The lack of response implied adrenal gland insensitivity to ACTH rather than lack of pituitary secretion of trophic hormone (Rossdale *et al.*, 1982). At

the beginning of this century, Toribio postulated that RAI is present in horses with colic, enterocolitis, sepsis, endotoxemia and DIC (Toribio, 2004).

A hallmark of RAI in humans is hypotension despite appropriate fluid and pressor therapy (Annane, 2005; Pizarro *et al.*, 2005). In a study of 10 septic foals, Gold, Divers and Bain reported that the 3/10 foals that were non-responsive to fluid therapy had extremely high single-sample ACTH concentrations with normal serum cortisol concentrations, suggesting dysregulation of the HPA axis. On the other hand, the 7/10 foals that were responsive to fluid therapy had elevated ACTH and cortisol concentrations, suggesting an appropriate HPA response to sepsis (Gold *et al.*, 2007).

In hospitalized horses subjected to necropsy, extensive adrenal gland haemorrhage, venous thrombosis and adrenocortical necrosis are common findings (Jubb *et al.*, 1985). The adrenal histopathologic lesions appear more severe in horses compared to other domestic species, and the adrenal gland is considered a "shock" organ for horses. In a 20 year career, I have had two critically ill horses die unexpectedly and adrenal gland haemorrhage was reported at necropsy as the cause of death. Adrenal haemorrhage and necrosis could be an acute event or could progress over a period of time. As adrenal exhaustion is likely to occur over time it appears necessary to monitor the HPA axis periodically throughout the hospitalisation period and not just at admission.

1.2.13 Diagnosis of adrenal dysfunction

The function of the HPA axis can be assessed by comparing cortisol concentrations to plasma CRH and ACTH concentrations. A high ACTH/cortisol ratio can be used to diagnose hypothalamic-adrenal dysregulation indicating adrenal exhaustion, adrenal damage or inadequate cortisol production and release in response to high pituitary output of ACTH. This is considered as a primary hypoadrenal status where there is low plasma cortisol and high ACTH. Secondary hypoadrenalism is distinguished by low cortisol and ACTH concentrations due to pituitary dysfunction. Hypothalamic dysfunction is considered tertiary adrenal insufficiency. In foals, just as in people, primary adrenocortical failure is the most common cause of adrenal insufficiency. In 2 populations of septic foals, non-survivors had significantly higher ACTH/cortisol ratios than surviving foals (Hurcombe *et al.*, 2008; Gold *et al.*, 2007; Bousquet-Melou *et al.*, 2006). High ACTH concentrations and low corresponding cortisol concentrations, suggest HPA dysfunction at the level of the adrenal gland, and therefore RAI.

1.2.14 Diagnosis of RAI and CIRCI in humans

Changes in plasma electrolyte concentrations or identification of peripheral blood eosinophilia as indicators of adrenal hypofunction lack sensitivity and specificity. Evaluation of adrenal function in critically ill patients relies on

either measurement of basal cortisol concentration or dynamic tests such as the ACTH stimulation test, metapyrone test, insulin hypoglycemic test or the CRH stimulation test. In normal humans resting cortisol concentrations are 10-20 µg/dL. The maximum stress induced cortisol in humans is approximately 60 µg/dL (Annane *et al.*, 1998). Mean basal cortisol in surviving human ICU patients were 27 µg/dL, compared to 47 µg/dL in non-survivors (Rothwell & Lawler, 1995). Others have found mean cortisol in human patients with septic shock to average 50 µg/dL (Marik & Zaloga, 2002). Due to the wide ranges of cortisol concentrations and severities of illness it is difficult to determine a concentration of cortisol that is adequate for every clinical situation. This is reflected in the reported incidences of adrenal insufficiency in critically ill patients which varies from 1.5-75% (Rai *et al.*, 2004). Although a single factor should never be used to prognosticate survival, a clear relationship has not been demonstrated between serum cortisol and mortality in critically ill patients (Rai *et al.*, 2004).

There is still a lack of consensus, even among a panel of experts, as to the best methods to diagnose CIRCI in humans (Annane *et al.*, 2018). Marik suggested that random cortisol concentrations < 25 µg/dL in highly stressed patients was useful to diagnose adrenal insufficiency and substantiate hydrocortisone therapy (Marik & Zaloga, 2003). The European CIRCI guidelines suggest using either a random endogenous cortisol of < 10 µg/dL (or a delta-cortisol of < 9 µg/dL after intravenous administration of 250 µg cosyntropin, which is the high-dose ACTH stimulation test) (Annane *et al.*, 2018). It can also vary dramatically throughout patient admission, resuscitation and over hospitalisation.

The insulin-induced hypoglycemic test is considered the gold standard to diagnose HPA dysfunction in humans. The administration of exogenous insulin results in hypoglycemia which is a potent stimulator of the HPA axis, therefore post insulin ACTH and cortisol are measured. However, the test is contraindicated in critically ill people due to glucose dysregulation that is often simultaneously occurring. The metyrapone test works by inhibition of adrenocortical 11 –βhydroxylase, which converts 11-deoxygotisol to cortisol. In normal individuals, the induced reduction in cortisol concentrations stimulates CRH and ACTH release and increases adrenocortical steroidogenesis and 11-deoxygotisol accumulation (Marik, 2009). A CRH stimulation test can also be performed to assess the pituitary adrenal response (secondary hypocortisolemia).

1.2.15 High dose and low physiological dose ACTH stimulation tests

Traditionally, to diagnose absolute adrenal insufficiency (Addison's disease), supra-physiological doses of ACTH were administered, e.g. 250 µg /human patient or ~2-5 µg/kg. However, in critically ill human patients, a normal response to this dose does not rule out RAI. RAI is believed to be due, in part, to adrenal resistance to ACTH. In humans, administration of 250 µg of ACTH

(100-fold greater than endogenous stress-induced ACTH levels (Oelkers, 1996) can override adrenal resistance to ACTH, resulting in a normal cortisol response, thus falsely ruling out a diagnosis of RAI. In human medicine, the physiologic low-dose (1-2 μg/patient) ACTH test appears more sensitive at diagnosing early/mild cases of adrenal suppression, and accordingly, has been recommended by some sources to replace the conventional 250μg test for identification of adrenal insufficiency in humans (Gonc *et al.*, 2003); Beishuizen *et al.*, 2000) including premature infants (Nykanen *et al.*, 1999). One randomized trial showed that the low-dose (1 μg) ACTH test was better able to predict the longer duration of vasopressor dependence and the hemodynamic response to hydrocortisone in septic shock patients than the high-dose ACTH test or a random basal cortisol < 25 μg/dL, in extremely ill people with a mortality rate of 47% (Marik & Zaloga, 2003). A prospective study with 74 septic shock patients showed that the high and low-dose ACTH stimulation tests were equally accurate in predicting dependency on vasopressors and mortality (Moraes *et al.*, 2012).

There is still a lack of consensus, even among a panel of experts, as to the best methods to diagnose CIRCI in humans (Annane *et al.*, 2018). The European CIRCI guidelines suggest using either a random endogenous cortisol of < 10 μg/dL or a delta-cortisol of < 9 μg/dL after intravenous administration of 250 μg cosyntropin (high-dose ACTH stimulation test) (Annane *et al.*, 2018). The main reason given for utilizing the high-dose ACTH stimulation test over the low-dose ACTH stimulation test for critically ill humans was simply that of convenience as the low-dose test requires dilution of the ACTH and calculation of an appropriate volume, while in a high dose test the entire 250 μg vial of ACTH is injected. The high dose test is efficacious and considered safe, even to perform on critically ill neonates, with the transient cortisol release considered of little consequence (and potential benefit) to the patient. However, in veterinary practice where the expense of diagnostic procedures is often the main limiting factor limiting their use, a low dose ACTH stimulation test would be significantly cheaper to perform than a high dose ACTH stimulation test. Although most studies show it is equally sensitive in diagnosing adrenal RAI in critically ill patients, there is also some evidence to suggest it is more sensitive at diagnosing adrenal dysfunction in this group (Moraes *et al.*, 2012; Marik & Zaloga, 2003).

1.2.16 Studies of the HPA axis in sick neonatal foals

Several recent studies have investigated the HPA axis in sick and septic foals (Dembek *et al.*, 2013; Wong *et al.*, 2009a; Hurcombe *et al.*, 2008; Gold *et al.*, 2007; Hart *et al.*, 2007). Adrenal dysfunction has been identified in septic neonatal foals by the use of a low-dose ACTH stimulation test (Hart *et al.*, 2009b; Wong *et al.*, 2009a). No significant differences in peak or delta cortisol response were detected between groups of ill and age-matched healthy foals in response to a low-dose ACTH stimulation test (0.1 µg/kg IV) (Wong *et al.*, 2009a) or to a paired low-dose (10 µg IV)/high-dose (100 µg IV) ACTH stimulation test (Hart *et al.*, 2009b). However, when human diagnostic criteria for CIRCI were utilized, approximately 50% of all hospitalized foals and approximately 40% of septic foals met these criteria (Hart *et al.*, 2009b). Non-surviving foals had significantly lower cortisol responses to low-dose ACTH stimulation as compared to survivors (Wong *et al.*, 2009a). Foals that had an inadequate cortisol response to a high-dose ACTH stimulation test had a significantly greater incidence of shock, multiple organ dysfunction syndrome, and non-survival than foals with an adequate cortisol response to ACTH (Hart *et al.*, 2009b).

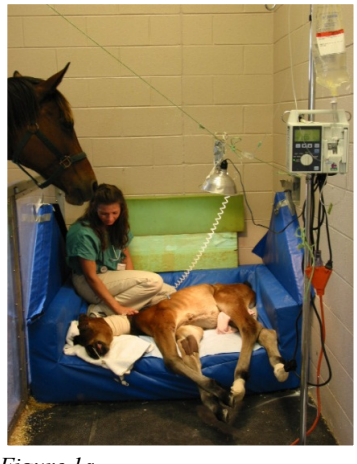

Figure 1a.

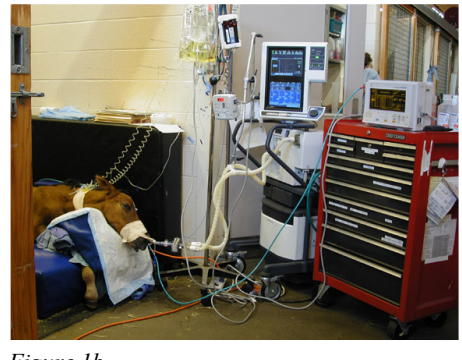

Figure 1b.

Figure 1a. Septic neonatal foal receiving intensive care
Figure 1b. Foal with botulism receiving positive pressure ventilation
Photos: A.J. Stewart.

1.2.17 Determination of HPA axis in sick adult horses

Cortisol concentrations have been measured in sick pregnant mares, (Santschi *et al.*, 1991) equine colic patients (Mair *et al.*, 2014; Ayala *et al.*, 2012;

Hinchcliff *et al.*, 2005; Hoffsis & Murdick, 1970). Sparse information is available for about ACTH concentrations in adult horses in association with illness (Mair *et al.*, 2014; Ayala *et al.*, 2012). Sick animals should have higher cortisol concentrations than healthy animals, but it is unknown what an appropriate stressed cortisol concentration should be with various severities of illness in horses. To simulate a physiologically stressed basal cortisol, Hart used the lowest (mean – 1 SD) cortisol concentration obtained after administration of cosyntropin to healthy foals to define a cut-off value for an appropriate basal cortisol concentration in sick foals (Hart *et al.*, 2009b). As adrenal exhaustion is likely to occur progressively over time it is highly relevant, as done in this thesis work to monitor the HPA axis periodically over the duration of hospitalisation. Serial monitoring of HPA function is frequently recommended but rarely performed and never reported in critically ill veterinary species. There are many studies that have performed ACTH

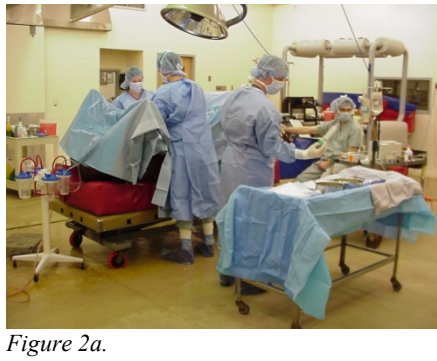

Figure 2a.

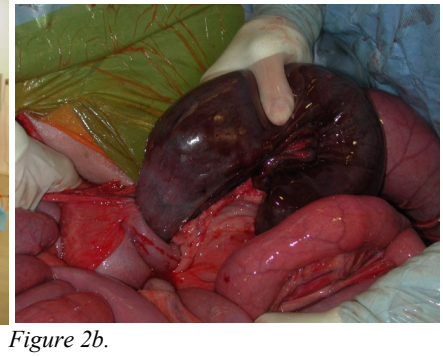

Figure 2b.

Figure 2a. Exploratory laparotomy being performed on an anaesthetised horse in dorsal recumbency.
Figure 2b. Ischaemic small intestine secondary to a strangulating lipoma from the horse undergoing exploratory laparotomy
Photos: A.J. Stewart.

stimulation tests at admission, (Wong *et al.*, 2009a; Martin *et al.*, 2008; Burkitt *et al.*, 2007) but to the authors knowledge there are no veterinary studies that have performed ACTH stimulation tests repeatedly throughout hospitalisation. Although many patients improve rapidly with intensive care, the effects of prolonged stress of severe illness, adrenal exhaustion or adrenal haemorrhage and necrosis may result in the development of RAI during the period of hospitalisation that was not identified during a single sample test at admission to the ICU. Cortisol concentrations are often remarkably (and appropriately) high at admission in equine colic patients, (Mair *et al.*, 2014; Hinchcliff *et al.*, 2005) yet after several days of critical illness, hypothalamic and adrenal function may well be severely compromised. Sequential monitoring, especially in patients that are not rapidly improving is therefore warranted.

1.2.18 Treatment of RAI

Although septic patients do not benefit from a short course of supra-physiological doses of corticosteroids, (Bone & Fisher, 1987) results of several recent randomized controlled trials in humans, indicate that administration of physiological doses can reduce the mortality associated with septic shock (Annane, 2001). In human patients diagnosed with RAI, a marked improvement in survival occurred after subsequent treatment with low "stress" doses of glucocorticoids (Annane *et al.*, 2002; Rivers *et al.*, 2001; McKee & Finlay, 1983). Moreover, glucocorticoid therapy does not increase the complication rate compared to placebo (Annane *et al.*, 2002).

Premature foals that have experienced in utero stress have a higher N/L ratio and a better prognosis for survival than prematurely delivered foals. Experimental direct administration of CRH, ACTH or betamethasone to the foetus in utero using ultrasound guided intramuscular injections in the last month of gestation resulted in increased foetal cortisol and elevated maternal progesterone levels consistent with foetal adrenal gland maturation. This procedure is not without risk and did result in abortion in a subset of mares. Multiple injections of depot corticotropin$_{1\text{-}24}$ to pregnant mares at risk of premature delivery as well as postpartum administration to premature foals appears to hasten hormonal maturity, but is still under investigation.(Silver & Fowden, 1994) In human patients diagnosed with RAI low dose of hydrocortisone (50-300 mg per day) over 5-7 days resulted in clinical improvement and increased survival (Annane, 2001). Recognition and treatment of CIRCI in human patients substantially improves the survival rate (Annane, 2005; McKee & Finlay, 1983).

1.2.19 Insulin and glucose regulation

The β cells of the endocrine pancreas are responsible for the synthesis and release of insulin, which is the main mammalian anabolic hormone. Insulin regulates carbohydrate and lipid metabolism by increasing glucose uptake from the blood to insulin sensitive tissues including skeletal muscle, adipose tissue and the liver. High circulating insulin concentrations promote the transformation of glucose into glycogen through the process of glycogenesis in the skeletal muscles and liver, or into triglycerides through lipogenesis in the adipose tissue and liver. There is a balance between glucose and insulin concentrations, with low of circulating insulin concentrations resulting in an increase in hepatic glucose secretion by promotion of gluconeogenesis and glycogenolysis (Sonksen & Sonksen, 2000).

1.2.20 Insulin resistance and the critically ill patient

Insulin resistance is defined as the failure of tissues to respond to endogenous or exogenous insulin, which results in uncontrolled hyperglycaemia (van den

Berghe *et al.*, 2001). Insulin resistance develops through several mechanisms at the cellular level. Cellular hypoxia results in decreased availability of the insulin receptor and oxidative stress leads to activation of inflammatory pathways and inhibition of post-receptor insulin signalling pathways.

In human patients with sepsis or severe trauma, peripheral tissue insulin resistance and hyperglycaemia are common. High blood glucose concentrations are associated with an increased risk of death and poor outcome (Griesdale *et al.*, 2009; Arabi *et al.*, 2008; van den Berghe *et al.*, 2001). Tight control of blood glucose concentrations between 80 – 110 mg/dL through intensive insulin therapy was associated with improved survival in critically ill patients (van den Berghe *et al.*, 2001). In contrast, results of other studies have demonstrated that intensive insulin therapy results in significant increases in the risk of hypoglycaemia, and is associated with poor survival, resulting in no overall change in mortality (Macrae *et al.*, 2014). Still other studies have shown that less stringent conventional glucose regulation, such as between 140 – 180 mg/dL through insulin infusions resulted in a decreased incidence of hypoglycaemia without an increase in mortality (Griesdale *et al.*, 2009; Preiser *et al.*, 2009; Arabi *et al.*, 2008). Guidelines suggest that cautious insulin therapy may be appropriate for critically ill patients.(Jacobi *et al.*, 2012)

1.2.21 Hyperglycaemia in sick horses and after lipopolysaccharide administration

Hyperglycaemia is common in horses with colic and is associated with non-survival (Hassel *et al.*, 2009; Hollis *et al.*, 2007). In the early stages of systemic inflammation, low serum insulin and increased insulin sensitivity has been shown in several studies involving horses (Tadros *et al.*, 2013b; Barsnick *et al.*, 2011; Barsnick *et al.*, 2010; Vick *et al.*, 2008). Tadros demonstrated decreases in insulin concentrations after lipopolysaccharide administration, suggesting that the resultant hyperglycaemia was from pancreatic dysfunction and peripheral insulin resistance (Tadros *et al.*, 2013). Little was known about insulin and glucose dynamics in adult critically ill horses and if insulin resistance or pancreatic dysfunction occurred in naturally occurring disease.

1.2.22 Measurement of hormone concentrations

Liquid chromatography and high-resolution/high-accuracy mass spectrometry (LC-MS) are the gold standards to measure hormone concentrations. Although useful in a research setting LC-MS is not utilized for clinical cases due to the requirement of sophisticated equipment and specialised technical skills and the time involved in sample analysis. Since there are no equine specific cortisol,

ACTH or insulin standards or antibodies, all commercially available assays will likely underestimate equine hormones compared to LC-MC. Radioimmunoassays (RIA), chemiluminescent immunoassays (CLIA) and enzyme-linked immunosorbent assays (ELISA) designed for use on human samples have been utilized for equine samples with variable levels of validation, co-efficients of variation, precision and accuracy. The RIA had been considered as the "gold standard"; however, because of the use of radioactive reagents, the tests becoming less popular. When concentrations of various hormones are measured using different analytical methodologies, it is difficult to directly compare actual values obtained in different studies. References ranges established at one laboratory may not be appropriate for another, especially when different assays are utilized.

Even using human samples, there is concern regarding the variations in precision and accuracy of cortisol assays. The co-efficient of variation between cortisol assays can vary by approximately 10-12% (Rai *et al.*, 2004). When one sample is submitted to multiple laboratories various assays can provide significantly different results (Clark *et al.*, 1998). In humans with septic shock immunoassays for total cortisol also appear to correlate poorly (inter-assay variations in up to 27% of cases), when compared to the gold standard of liquid–chromatography mass spectrometry (LC-MS) (Bornstein, 2009; Briegel *et al.*, 2009). Although there are limitations in the use of immunoassays to measure cortisol and ACTH concentrations, standardized sample handling procedures for samples and looking at trends and relative changes in hormone concentrations measured on same assay from the same institution are likely to be valid. The ELISA has been used in many research projects for measurement of insulin, but the test uses porcine standards and provides lower insulin values than the RIA. The CLIA is commonly used in both clinical and research settings; but the assay is based on human cortisol, insulin and ACTH.

Although equine cortisol concentrations are fairly consistent between different assays, measurement of equine $ACTH_{1-39}$ has shown many inconsistencies between assays. In a large study in normal and PPID horses, Banse et al, found poor agreement when comparing one particular radioimmunoassay (RIA kit, Biomedicals, Orangeburg, NY) with the chemiluminescent Immulite™ assay with large discordance between results.(Banse *et al.*, 2018) In a smaller study evaluating 39 plasma samples from normal and PPID-affected horses, when evaluating the Scantibodies™ immunoradiometric kit compared to various other radioimmunoassays and the chemiluminescent Immulite™ assay, Schott et al found fairly good correlation between the Scantibodies™ and Immulite™ assays. However the Immulite™ yielded ACTH concentrations 3.6-fold greater than the Scantabodies™ assay, with Bland-Altman analysis revealing bias ± limits of agreement of 24.2 ± 62.9 between these two assays. There was a positive bias of the Immulite™ increasing with increasing ACTH concentrations in PPID horses (Schott, 2013). As $ACTH_{1-39}$ produced in times of stress originates from the corticotropes of the pars distalis, rather than pars intermedia melanotrope derived ACTH in PPID-

affected horses, where incomplete ACTH fragments and other pro-opiomelanocortin (POMC) derived peptides may be measured as ACTH, the consequences of studies comparing assays of immunoreactively measurable ACTH may not be as of great a consequence in sick horses compared to PPID-affected horses.

2 Aims of the thesis

The overall aim for this thesis was to study factors that influence cortisol and ACTH dynamics and insulin and glucose dynamics in sick adult horses after validating the ACTH stimulation test in adult horses and neonatal foals.

The specific objectives were:

- To validate the ACTH stimulation test in adult horses and neonatal foals.
- To determine the lowest ACTH dose that would consistently induce a significant increase in cortisol concentration in healthy adult horses and neonatal foals.
- To determine the lowest ACTH dose required to induce a maximum increase in cortisol concentration and to identify the time to peak cortisol concentration after administration of low doses of ACTH.
- To determine the effect of administration of low doses of ACTH on haematologic values in healthy adult horses and neonatal foals.
- To describe the glucose and insulin dynamics in horses diagnosed with SIRS and evaluate their usefulness as predictors of survival.
- To determine if ACTH and cortisol concentrations, and the ACTH/cortisol ratio vary with survival, presence of SIRS or ischemic gastrointestinal lesions at admission, or throughout hospitalisation.
- To determine if sick horses had inappropriately low cortisol concentrations before and after an ACTH stimulation test.

3 Comments on materials and method

This section provides a brief summary on materials and methods used in the separate studies (I – V) included in this thesis. More detailed descriptions are found in each of the papers.

3.1 Horses

All aspects of studies I and II were approved by the Auburn University Institutional Laboratory Animal Care and Use Committees (20040744). The adult horses and the majority of foals used in the studies were owned by the Department of Clinical Sciences (Figure 3a), College of Veterinary Medicine, Auburn University, Auburn, Alabama, USA. Some foals were client owned and were lent to me for the purpose of sampling for this project.

All aspects of the studies III - V were approved by the Auburn and Colorado State Universities Institutional Laboratory Animal Care and Use Committees (20091564, 09-079A-01) and the College of Veterinary Medicine Clinical Research Review Committee, with signed owner's consent obtained for ACTH stimulation tests. The dose of ACTH used on the sick horses was 0.1 µg/kg which is less than the high dose of 250 µg/patient that is routinely administered to critically ill human patients, including septic infants. The transient rise in cortisol concentrations induced are not considered detrimental in any manner and there have been no side effects reported from the test. The horses were privately owned patients admitted on an emergency basis to the John Thomas Vaughan Large Animal Teaching Hospital, College of Veterinary Medicine, Auburn University, USA and Colorado State University, College of Veterinary Medicine & Biomedical Sciences, Fort Collins, USA. Information provided to clients regarding horse enrolment is included in appendix 1. The client consent form is in appendix 2.

Horses with clinical signs of Equine Metabolic Syndrome (EMS) and PPID were excluded from each study. Horses and foals in studies I and II were deemed to be clinically healthy based on history, physical examination findings, and results of a complete blood count (CBC), serum biochemical profile and

fibrinogen concentration. No horse had received any medication in the preceding 2 months.

For projects III – V sick horses presenting to the equine emergency service of two university teaching hospitals (Auburn University and Colorado State University) over two summers (May to August) were enrolled (Figure 3b). For project III only horses ≥ 5 years that were diagnosed with SIRS were included, while in studies IV – V horses ≥ 1 year were included. Data recorded included signalment, clinical examination findings and routine admission bloodwork (haematological and biochemical data); diagnosis and duration of hospitalisation.

For sick horses blood was drawn through an intravenous catheter placed for the purposes of treatment. The study had no influence on clinical decisions or treatments administered to patients.

Figure 3a.

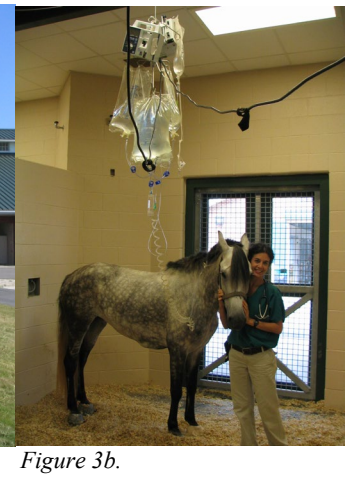

Figure 3b.

Figure 3a. University owned brood mare and foal used in project III and later adopted as a potential sport horse.
Figure 3b. Client owned horse in the equine intensive care unit receiving intravenous fluids after having 2 meters of small intestine removed surgically the previous evening.
Photos: A.J. Stewart.

3.2 Study designs

3.2.1 Study I

One of the previous difficulties in trying to diagnose RAI in horses was that the ideal dose of ACTH required for ACTH stimulation testing was unknown. Therefore, we performed a study to determine the lowest dosage of ACTH

required to produce a significant increase in cortisol concentrations in normal adult horses (Stewart *et al.*, 2011). In a randomized crossover design, 5 ACTH stimulation tests were performed on each of 8 horses (4 mares and 4 geldings), with a 3-day wash-out period between tests. Four dosages (0.02, 0.1, 0.25 and 0.5µg/kg) of cosyntropin and saline as a negative control were administered IV into a jugular vein. Blood samples were collected before (0 minutes [baseline]) and 30, 60, 90, 120, 180, and 240 minutes after injection of cosyntropin or saline solution for measurement of serum cortisol at each time point and plasma ACTH at baseline. Complete blood counts were performed with an automated analyser (Advia 120 Haematology System, Siemens, Tarrytown, NY.) and differential cell counts were performed by hand at 0, 60, 120, and 240 minutes after injection of cosyntropin or saline solution for routine CBCs.

3.2.2 Study II

Eleven apparently healthy foals (6 colts and 5 fillies) were studied. All foals were full term (> 330 days gestation), born without assistance and had an immunoglobulin concentration (> 800 mg/dL or < 8 g/L) by 24 hours of age. Foals entered the study at 2 days of age and were 12 days of age by study completion. No foal had received any medication prior to the study except diazepam and butorphanol for placement of a central venous catheter the day prior to study commencement (Figure 3c). The foals weighed from 32 to 65 kg; 52 ± 11 kg at the commencement of the study and were weighed daily during the study. Foals gained 4.5 to 29 kg13 ± 7 kg by the end of the study. In a randomized crossover design, 5 ACTH stimulation tests were performed on each foal, with a 2-day washout period between tests. For each test, saline (0.9% NaCl) solution or 1 of 4 doses (0.02, 0.1, 0.25, and 0.5 µg/kg cosyntropin was administered IV into the contralateral jugular vein via a transiently placed catheter. Blood samples were collected from the central venous jugular catheter immediately before (0 minutes [baseline]) and 30, 60, 90, 120, 180, and 240 minutes after injection of cosyntropin or saline solution for measurement of cortisol concentration each time point and plasma ACTH at baseline. Samples were also collected at 10 and 20 minutes in 6 of 11 foals for cortisol measurement. Blood was also collected before and 60, 120, and 240 minutes after injection of cosyntropin or saline solution for performance of a routine CBC.

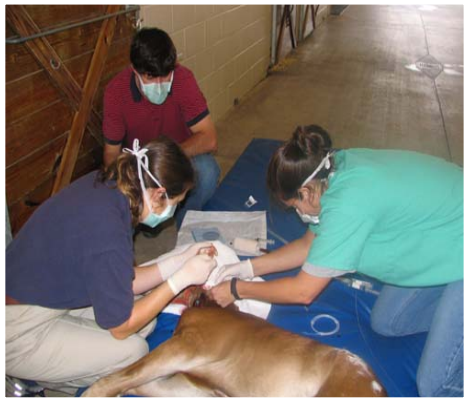

Figure 3c. Placing a central venous catheter in a heavily sedated 1 day old foal for later collection of blood samples
Photo: A.J. Stewart.

3.2.3 Study III

To investigate insulin dysregulation in horses with SIRS a subset of horses used in study IV that presented to the emergency service of the Auburn University J.T. Vaughan Large Animal Teaching Hospital that fit additional criteria of being > 5 years of age and diagnosed with SIRS were included. Serum insulin and glucose concentrations were measured at admission from archived frozen serum samples. Insulin concentrations were measured in duplicate using an radioimmunoassay and blood glucose was measured using a glucohexokinase colorimetric assay as previously described (Reimers *et al.*, 1982) (de Laat *et al.*, 2016). Horses were grouped by outcome (survival, hyperinsulinemia and hyperglycaemia).

3.2.4 Study IV

To determine the cortisol and ACTH concentrations and ACTH/cortisol ratios in sick horses throughout hospitalisation and any associations with survival, an ischemic gastrointestinal lesion and SIRS. One hundred and fifty-one horses (> 1 year of age) presenting to the emergency service of two university teaching hospitals (Auburn University and Colorado State University) were recruited over two summers (mid-May until mid-August) and serum cortisol and plasma ACTH concentrations measured at admission and on morning of days 2, 4 and 6 if the patient was still hospitalized. Data recorded included signalment, physical examination findings and routine bloodwork at admission (haematological and biochemical data); diagnosis, outcome, duration of hospitalisation, diagnosis of SIRS, SIRS score (see below) and presence of an ischemic gastrointestinal lesion

for horses that underwent surgery or necropsy. For statistical analysis horses were grouped by outcome (survival, SIRS score, presence of SIRS, SIRS score and an ischemic lesion).

3.2.5 Study V

To identify adrenal dysfunction in critically ill adult horses, a subset of 58 patients enrolled for project IV were requested to be involved in ACTH testing. Within the first 2 hours of admission, blood was collected for baseline endogenous serum cortisol and plasma ACTH concentrations. A low dose ACTH stimulation test was then performed with administration of 0.1 µg/kg IV of previously diluted cosyntropin with blood was collected at 30 minutes (T = 30) for post ACTH cortisol measurement, as previously described (Stewart *et al.*, 2011). This sampling occurred at admission and on the mornings of days 2, 4 and 6 if the patient was still hospitalized, having not been discharged or euthanized. Data recorded included signalment, physical examination findings and routine bloodwork at admission (haematological and biochemical data); diagnosis, outcome, duration of hospitalisation, diagnosis of SIRS, SIRS score and presence of an ischemic gastrointestinal lesion for horses that underwent surgery or necropsy. Criteria adapted from definitions utilized in human intensive care and in a study involving sick neonatal foals was used to define an inadequate adrenal response to the degree of illness (Annane *et al.*, 2018; Hart *et al.*, 2009b; Annane *et al.*, 2006). An inappropriately low basal cortisol concentration was defined by 2 methods: 1) a physiologic stressed cortisol concentration calculated as lowest cortisol concentration (mean - 1SD) obtained after administration of a physiologic dose (0.1 µg/kg) of cosyntropin to healthy adult horses which calculates as an endogenous cortisol <9.7 µg/dL and 2) a more conservative value of less than the lowest basal cortisol concentration (mean - 1SD) obtained from healthy horses, which calculates as < 4.51µg/dL (Hart *et al.*, 2011; Stewart *et al.*, 2011). An inappropriately low delta-cortisol 30 minutes after the administration of 0.1 µg/kg of cortrosyn was defined by two methods: 1) a value less than the mean delta-cortisol obtained from healthy horses which was < 5.65 µg/dL (definition previously utilized in a study of neonatal foals) and a more conservative value less than the mean delta-cortisol-1SD obtained from healthy horses which was < 4.15 µg/dL (Hart *et al.*, 2011; Stewart *et al.*, 2011). A horse was defined as having RAI/CIRCI if it had an inappropriately low basal or delta-cortisol and it was considered to be critically ill based on clinical parameters. Horses were grouped by outcome (survival, SIRS score, presence of SIRS and an ischemic lesion).

3.3 Blood collection and sample handling

Blood was collected through an aseptically placed intravenous catheter (Figures 4a-d) or by venipuncture and placed in a plain glass tube and a cold tube

containing EDTA mixed with 0.3 mL of the protease inhibitor aprotinin (lyophilized powder, USB Corp, Cleveland) with final concentration of 500 kallikrein inactivator units/mL of blood (Kemppainen *et al.*, 1994). Plasma was centrifuged within 20 minutes, while serum was collected by centrifugation after allowing the blood sample to clot for 45 minutes at room temperature. Samples were frozen at -80°C until assayed.

Figure 4a

Figure 4b

Figure 4c

Figure 4d

Figure 4a Student assistants collecting blood from a jugular catheter on a sick horse for project III, IV or V
Figure 4b Student assistant placing blood into plain and EDTA vacutainer tubes
Figure 4c Client consent form, diluted cosyntropin and blood tubes in preparation for performing a low-dose ACTH stimulation test for project V.
Figure 4d Student assistants collecting blood from a jugular catheter from a university owned research foal for project II.
Photos: A.J. Stewart.

3.4 Cosyntropin use in the ACTH stimulation test

For projects I, II and V lyophilized cosyntropin (synthetic ACTH; Cortrosyn®, Amphastar Pharmaceuticals Inc, Rancho Cucamonga, Calif), was diluted with 1.0 mL of sterile saline solution in accordance with the manufacturer's instructions. Cosyntropin was then further diluted with sterile saline solution to a final concentration of either 10 µg/mL (for administration of the 0.25 and 0.5 µg/kg doses) or 1 µg/mL (for administration of the 0.02 and 0.1 µg/kg doses). Unused reconstituted cosyntropin solution (1 µg/mL) was refrigerated in the original plastic saline solution bag for use within the next week. Diluted cosyntropin solution has been shown to remain fully stable (in concentrations as low as 0.5 µg/mL) for at least 4 months when refrigerated in plastic containers.[40]

3.5 Radioimmunoassays

Cortisol, ACTH and insulin concentrations were measured in duplicate using radioimmunoassays designed for analysis of human hormones (Figure 5). The Coat-A-Count cortisol direct radioimmunoassay (In-vitro Diagnostic Test Kit, Diagnostic Products Corporation, Los Angeles, CA) had been previously validated in horses and was used in all studies I, II, IV and V (Funk *et al.*, 2011; Golland *et al.*, 1999). The inter-assay and intra-assay coefficients of variation ranged from 7.1% to 7.7% and from 6.1% to 8.1%, respectively for cortisol. The laboratory reference interval for cortisol concentration in normal horses was 1.49 – 6.45 µg/dL (Schreiber *et al.*, 2012).

In projects I and II plasma endogenous ACTH concentration was measured by use of a sandwich immunoradiometric assay previously validated for use in horses (Adrenocorticotropic hormone immunoradiometric assay, Nichols Institute, San Clemente, Calif.) (Marc *et al.*, 2000; Golland *et al.*, 1999). The inter-assay and intra-assay coefficients of variation were, for values < 30 pg/mL, 9.2% and 4.5%, respectively (Marc *et al.*, 2000). For projects IV and V the ACTH immunoradiometric assay from Scantibodies Laboratory Inc, Santee, CA had intra- and inter-assay coefficients of variation of < 15% and < 19%, respectively (Schreiber *et al.*, 2012; Funk *et al.*, 2011). The upper limit of the laboratory reference interval was 28 pg/mL for the summer period (Schreiber *et al.*, 2012; Funk *et al.*, 2011).

Equine serum insulin was measured using a radioimmunoassay previously validated in horses. The Coat-A–Count cortisol direct radioimmunoassay, (Diagnostic Products Corp, Los Angeles, CA) intra- and inter-assay variation: 5.2% and 6.4% respectively) as previously described (Reimers *et al.*, 1982)·(de Laat *et al.*, 2016).

Figure 5 Morris Animal Foundation Veterinary Summer Scholarship students assiting with sample preparation for measuring hormone concnetrations using radioimmunoassays. All students under went laboratory radiation safety training Photos: A.J. Stewart.

3.6 Definition of SIRS and SIRS Score

The diagnosis of SIRS was based on the presence of 2 or more of the following criteria: hyperthermia (>38.6°C) or hypothermia (<36.1°C), tachypnoea (> 24 breaths/minute), tachycardia (> 45 beats/minute), > 10% band neutrophils, leukopenia (white cell count < 6,000/µL) or leucocytosis (white cell count > 12,000/µL).(Arroyo *et al.*, 2017) SIRS scores were defined by the number of SIRS criteria. Horses with any two SIRS criteria were allocated to group 2; any three SIRS criteria in group 3, etc. For example, a horse presenting with pyrexia and leukopenia would be in group 2, while a horse with pyrexia, tachypnoea, tachycardia and leucocytosis would be in group 4 (Bertin *et al.*, 2018a).

3.7 Clinicopathological data collected (III-V)

For projects III –V the data recorded included signalment, physical examination findings and routine bloodwork at admission (haematological and biochemical data). Signalment data included gender, age and breed. Presenting physical examination findings recorded included rectal temperature (°C), heart rate (beats/minute), respiratory rate (breaths/minute), mucous membrane colour (grades 0-3), estimated percent dehydration (%), capillary refill time (seconds) and rectal examination findings. Presenting haematological data recorded included white blood cell count (1000 cells/µL), neutrophil count (1000 cells/ µL), lymphocyte count (1000 cells/µL), band neutrophil count (1000 cells/µL), packed cell volume (%). Presenting biochemical data recorded included total protein (g/dL), albumin (g/dL), creatinine (mg/dL), blood urea nitrogen (mg/dL, lactate, fibrinogen (mg/dL), sodium (mEq/L), chloride (mEq/L), potassium (mEq/L), bicarbonate (mEq/L), glucose (mg/dL), venous base excess (mEq/L), total calcium (mg/dL), ionized calcium (mmol/L), total magnesium (mg/dL), ionized magnesium (mmol/L). Abdominocentesis data recorded included total protein (g/dL), specific gravity, white cell count (1000 cells/ µL) and cytological description.

The mean heart rate (beats/minute) and daily total volume of reflux on each day of hospitalisation (litres) was also recorded. Surgical or necropsy findings (where appropriate), length of hospitalisation and total bill ($USD) were tabulated. A severity of illness score (1 to 3) and pain score (1-3) was recorded for admission and overall based on clinical judgement.

3.8 Statistical analysis

3.8.1 Project I

The results were presented as mean ± SD. For each time point in each horse, delta cortisol concentration was calculated as cortisol concentration measured at that time minus the baseline cortisol concentration. Differences in endogenous cortisol and ACTH concentrations, ACTH-stimulated cortisol concentrations, delta cortisol concentrations, and CBC results over time were analysed by means of repeated-measures ANOVA followed by the least significant difference test. Analyses were performed with standard software. (SAS software, version 9.1, SAS Institute Inc, Cary, NC.) The dose effect at each time point was determined by comparison of the ACTH-stimulated cortisol concentration with the cortisol concentration measured after administration of saline solution. The cortisol concentration measured at each time point, for each cosyntropin dose, was

compared with the baseline cortisol concentration. Baseline endogenous serum cortisol and plasma ACTH concentrations were also compared among days that samples were collected. Differences in maximum cortisol concentration and time of maximum cortisol concentration between doses were determined by using the Friedman repeated-measures test, with post hoc comparisons performed using the Student-Newman-Keuls method (SigmaStat for Windows, version 1.0, Jandel Scientific, SPSS Inc, Chicago, Ill.) For all analyses, values of $P < 0.05$ were considered significant.

3.8.2 Project II

Results are summarized as mean ± SD values. For each time point after baseline for each foal, delta cortisol concentration was calculated as cortisol concentration measured at that time minus the baseline cortisol concentration. Differences in ACTH-stimulated cortisol concentrations, delta cortisol concentrations, and CBC results were analyzed via the mixed model for 2-D (time and dose) repeated-measures ANOVA and the Scheffe test for multiple comparisons. Analyses were performed with standard software (SAS software, version 9.1, SAS Institute Inc, Cary, NC.). The dose effect at each time point was determined by comparing the ACTH-stimulated cortisol concentration with the cortisol concentration measured after administration of saline solution. For each cosyntropin dose, cortisol concentration measured at each time point was compared with the baseline concentration. Baseline endogenous serum cortisol and plasma ACTH concentrations were also compared among days that samples were collected. Differences in maximum cortisol concentration and time of maximum cortisol concentration between doses were determined. For all analyses, values of $P < 0.05$ were considered significant.

3.8.3 Project III

Horses were categorized based on survival, hyperinsulinemia, hyperglycaemia and number of SIRS criteria and compared with $P < 0.05$ considered statistically significant. Normality was assessed by a Shapiro-Wilk normality test. Data following normal distribution were reported as mean ± SD and compared with an unpaired t-test, whereas data not following a normal distribution were reported as median [range] and compared using a Mann-Whitney U-test. A receiver operating characteristic (ROC) curve was plotted to analyse the prognostic value of a given variable (serum insulin, serum glucose or ratios). When more than 2 groups were compared, an ANOVA was used for normally distributed data and a Kruskal-Wallis test was used for non-normally distributed

data with a Dunn's post hoc test when appropriate. Categorical data were reported as counts and percentage of horses in which the variable was documented and compared using either a Chi-square test or a Fisher's exact test depending on expected counts. Odds ratios and 95% confidence intervals were calculated when appropriate. Statistical analysis was performed using commercially available statistical software (Prism, GraphPad Software, Inc. La Jolla, CA 92037, USA).

3.8.4 Project IV

Horses were categorized based on survival (discharged alive or not), presence of SIRS (≥ 2 SIRS criteria), number of SIRS criteria (SIRS score) and presence of an ischemic gastrointestinal lesion and compared, with $P < .05$ considered statistically significant. Normality was assessed by a Shapiro-Wilk normality test. A receiver operating characteristic (ROC) curve was plotted to analyse the prognostic value of a given variable (ACTH, cortisol or ACTH/cortisol ratio) at admission for predicting survival, presence of an ischemic lesion or SIRS. A two-way repeated measures ANOVA was used to determine the effect of survival, SIRS or ischemia and time and Sidak's multiple comparisons test was used for post hoc analysis. Data following a normal distribution were reported as mean ± SD values and data not following a normal distribution were reported as median [range]. Categorical data were reported as counts and percentage of horses in which the variable was documented and compared using either a Chi-square test or a Fisher's exact test depending on expected counts. Odds ratios (OR) and 95% confidence intervals were calculated when appropriate. Any groups with low numbers of data were excluded from statistical analysis. Commercially available statistical software was used (Prism, GraphPad Software, Inc. La Jolla, CA 92037, USA).

3.8.5 Project IV

The statistical analysis was identical to project IV except for the following: For admission data, data following normal distribution were compared with an unpaired t-test or a one-way ANOVA, depending on the number of groups compared, whereas data not following a normal distribution were compared using a Mann-Whitney U-test or a Kruskal-Wallis test, depending on the number of groups compared. To compare data during hospitalisation, a two-way repeated measures ANOVA was used to determine the effect of outcome of interest (survival, SIRS and ischemia) and time using data from horses that had a complete set of data.

4 Main Results

This section summarizes the main results from the separate studies. More detailed descriptions are presented in each of the papers (I –V). Figures and tables referred to the respective published papers and manuscripts are located at the end of the thesis

4.1 Project 1: Validation of a low-dose ACTH stimulation test in healthy adult horses

4.1.1 Baseline cortisol and ACTH concentrations

Baseline endogenous plasma ACTH concentrations in the 40 samples obtained prior to the 5 tests performed in the 8 horses ranged from (mean ± SD, and from 6 to 37 pg/mL (19.2 ± 5.6 pg/mL). Baseline endogenous serum cortisol concentrations ranged from 76 to 264 nmol/L (mean ± SD, 172.4 ± 44.8 nmol/L) [2.7 to 9.6 µg/dL (6.2 ± 1.62 µg/dL)]. Mean baseline serum cortisol and plasma ACTH concentrations did not differ between test days. No significant increases in serum cortisol concentration were detected after administration of saline solution; however, there was a significant decrease in serum cortisol concentration 240 minutes after administration of saline solution, compared with the baseline concentration (Figure 6).

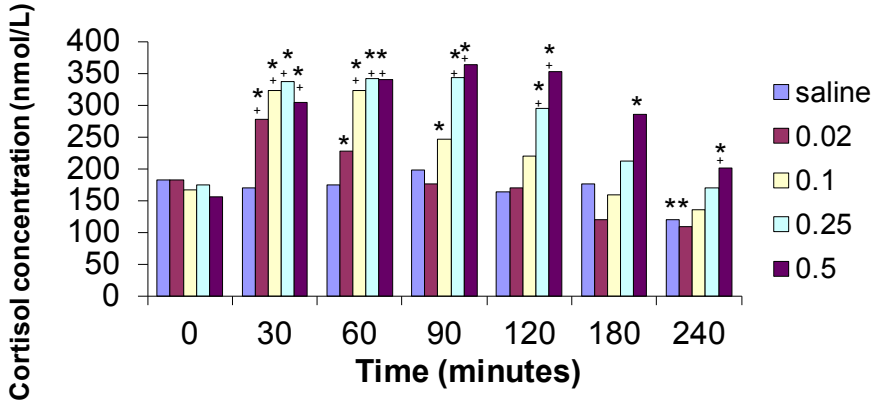

Figure 6. Mean serum cortisol concentration in 8 healthy adult horses given saline (0.9% NaCl) solution or 1 of 4 doses (0.02, 0.1, 0.25, and 0.5 μg/kg [0.009, 0.045, 0.114, and 0.227 μg/lb]) of cosyntropin. +Significantly ($P \leq 0.05$) different from concentration measured at the same time point after administration of saline solution. *Significantly ($P \leq 0.05$) different from baseline (time 0) concentration for that dose.

4.1.2 Cortisol concentrations after ACTH stimulation

For all 4 doses of cosyntropin, serum cortisol concentrations were significantly increased, compared with baseline concentrations at T = 30 and 60 minutes after cosyntropin administration, and significantly increased compared with concentration at T = 30 minutes after administration of saline solution. After ~~a~~ cosyntropin at a dose of 0.02 and 0.1 μg/kg, cortisol peaked at T = 30 and was 1.5 and 1.9 times the baseline concentration (Table 1 of paper I). After a cosyntropin dose of 0.25 and 0.5 μg/kg, cortisol peaked at 90 minutes and was 2 and 2.3 times baseline.

A maximum adrenal response was obtained between 30 and 90 minutes after administration of cosyntropin at a dose of 0.1 μg/kg, between 30 and 120 minutes after administration of cosyntropin at a dose of 0.25 μg/kg, and between 30 and 180 minutes after administration of cosyntropin at a dose of 0.5 μg/kg.

4.1.3 Delta cortisol concentrations after ACTH stimulation

No significant differences were detected among delta cortisol concentrations at T = 30 at a dose of 0.02 μg/kg and delta cortisol concentrations at T = 30 and 60 minutes at a dose of 0.1 μg/kg (Table 2 of paper I). Delta cortisol peaked at T = 90 minutes at a dose of 0.25 or 0.5 μg/kg, and for both doses, peak delta cortisol concentration was significantly higher than obtained after the 2 lower doses.

4.1.4 Haematological changes after ACTH stimulation

Significant increases in WBC count, neutrophil count, and the neutrophil/lymphocyte ratio and decreases in eosinophil count were detected (Table 3 of Paper I). Timing and magnitude of the changes were dependent on the cosyntropin dose. There was a doubling of the neutrophil/lymphocyte ratio; compared with the baseline ratio at T = 240 minutes after administration of the 0.25 or 0.5 μg/kg doses, although this was only significant at the higher dose. For all 4 doses of cosyntropin, there were no significant differences in lymphocyte count, monocyte count, heamatocrit, or RBC count over time.

4.2 Project II: Validation of a low-dose ACTH stimulation test in healthy neonatal foals

4.2.1 Baseline cortisol and ACTH concentrations

Baseline endogenous plasma ACTH concentrations in the 55 samples obtained prior to the 5 tests performed in the 11 foals ranged from 7 to 33 pg/mL (16.6 ± 6.3 pg/mL). Baseline endogenous serum cortisol concentrations ranged from 14 to 149 nmol/L (mean ± SD, 52.7 ± 27.3 nmol/L) [0.51 to 5.4 μg/dL (1.9 ± 0.99 μg/dL) Mean baseline serum cortisol and plasma ACTH concentrations did not differ among test days.

4.2.2 Cortisol concentrations after ACTH stimulation

A significant increase in cortisol concentration was not detected after administration of 0.02 μg/kg of cosyntropin; however, significance was approached when comparing T = 0 to T = 20 (P = 0.061) and 30 minutes (P = 0.067; Figure 7). After the 0.1 and 0.25 μg/kg doses of cosyntropin, cortisol was significantly (P = 0.02 to < 0.001) increased, compared with baseline

concentrations at T = 10, 20 and 30 minutes. For the 0.5 µg/kg dose, serum cortisol concentration was significantly (P = 0.002 to < 0.001) increased, compared with baseline concentrations between 10 and 60 minutes.

Cortisol peaked at T = 20 minutes after administration of doses of 0.02, 0.1, and 0.25 µg/kg, with peak concentrations 1.7, 2.0, and 1.9 times baseline concentrations, respectively. Cortisol peaked at T = 30 minutes after the 0.5 µg/kg dose, with peak concentration 2.2 times baseline. No significant differences were detected in peak cortisol concentrations after the 0.25 µg/kg dose at 20 and 30 minutes and the 0.5 µg/kg dose at 30 minutes. After the 0.25 and 0.5 µg/kg doses, no significant differences were detected among serum cortisol concentrations at 10, 20, 30, and 60 minutes.

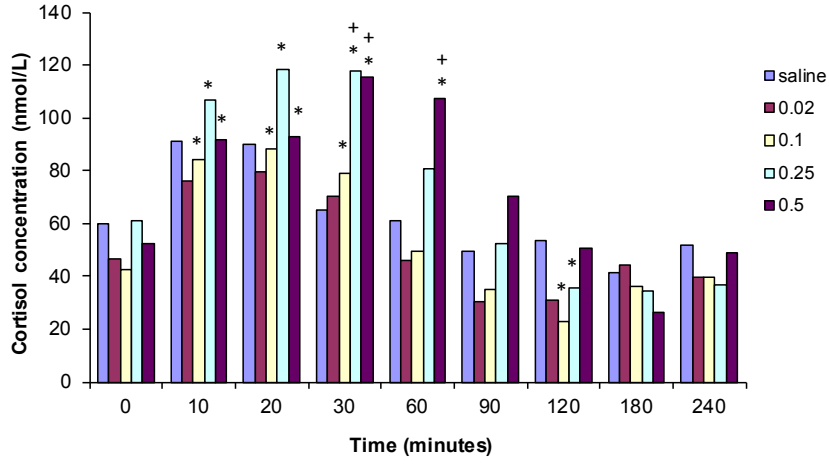

Figure 7. Mean serum cortisol concentrations in 11 healthy neonatal foals administered saline (0.9% NaCl) solution or 1 of 4 doses (0.02, 0.1, 0.25, and 0.5 µg/kg [0.009, 0.045, 0.114, and 0.227 µg/lb]) of cosyntropin. +Significantly ($P \leq 0.05$) different from concentration measured at the same time point after administration of saline solution. *Significantly ($P \leq 0.05$) different from baseline (time 0) concentration for that dose.

4.2.3 Comparison of cortisol response between cosyntropin doses and saline solution

After administration of saline, no significant differences were detected between cortisol concentrations at any time point or between cortisol concentrations after any of the cosyntropin doses, compared with cortisol concentrations subsequent

to saline solution administration at T = 10 and 20 minutes. There was a significant ($P < 0.001$) difference among cortisol concentrations after administration of saline solution compared to cortisol after the 0.25 and 0.5 µg/kg doses at T = 30 minutes and after the 0.5 µg/kg dose at T = 60 minutes ($P < 0.001$).

4.2.4 Delta cortisol response to various doses of cosyntropin

After the administration of the saline solution there were no significant differences between any of the delta cortisol concentrations (Figure 8). Delta cortisol concentrations at T = 10, 20, and 30 minutes were significantly different after administration of the 0.1, 0.25, and 0.5 µg/kg doses of cosyntropin and at T = 60 minutes after the administration of the 0.5 µg/kg dose compared with administration of saline. Delta cortisol peaked at T = 20 minutes after the 0.1 µg/kg dose, which was not different from the delta cortisol concentrations at T = 10 and 30 minutes. The peak delta cortisol concentration after administration of 0.25 µg/kg occurred at 30 minutes but was not different from the delta cortisol concentrations at 10 and 20 minutes. Delta cortisol concentration peaked at T = 30 minutes after the 0.5 µg/kg dose, and was not different from the delta cortisol concentrations at 10, 20, and 60 minutes.

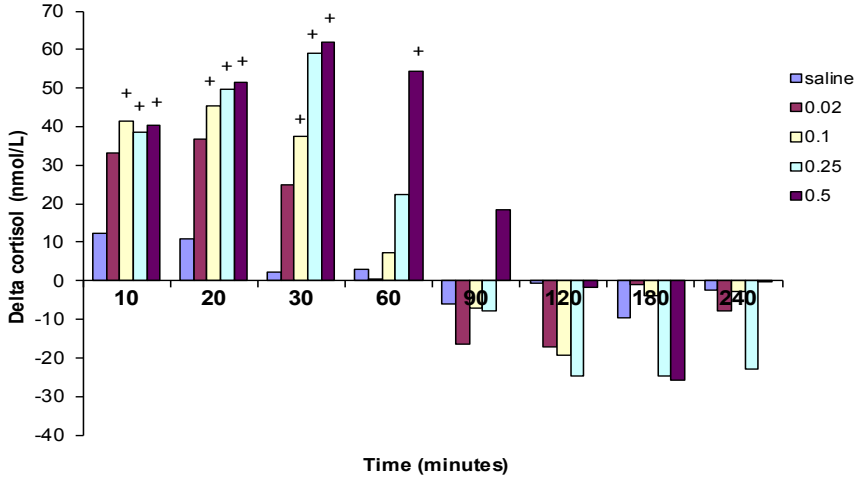

Figure 8. Mean delta cortisol concentrations in 11 healthy neonatal foals administered saline (0.9% NaCl) solution or 1 of 4 doses (0.02, 0.1, 0.25, and 0.5 µg/kg [0.009, 0.045, 0.114, and 0.227 µg/lb]) of cosyntropin. +Significantly ($P \leq 0.05$) different from concentration measured at the same time point after administration of saline solution.

4.2.5 Effect of cosyntropin on haematological variables

The neutrophil/lymphocyte (N/L) ratio increased 2.4-fold, from 4.76 ± 1.71 at baseline to 11.6 ± 11.6 at T = 120 minutes (P = 0.01). The lymphocyte count decreased from 1,555 ± 498 cells/µL at baseline to 1,122 ± 600 cells/µL at T = 120 minutes (P = 0.04) after the 0.25 µg/kg dose. For all 4 doses of cosyntropin, no significant differences in neutrophil count, monocyte count, eosinophil count, haematocrit or RBC count over time were detected. There were no changes in any parameters after administration of saline.

4.3 Comparison of Project I to Project II

4.3.1 Comparison of cortisol response to administered doses of cosyntropin between foals and adult horses

Adult horses had slightly higher baseline ACTH concentrations and much higher baseline cortisol concentrations (Figure 9a) than neonatal foals (Figure 9b). The cortisol response to 4 identical doses of cosyntropin administered to the 11 foals compared to that of 8 adult horses (Figure 2 of paper II) demonstrated that the ACTH-stimulated cortisol concentrations peaked later, were of higher magnitude, and were of a longer duration in adult horses, compared with foals.

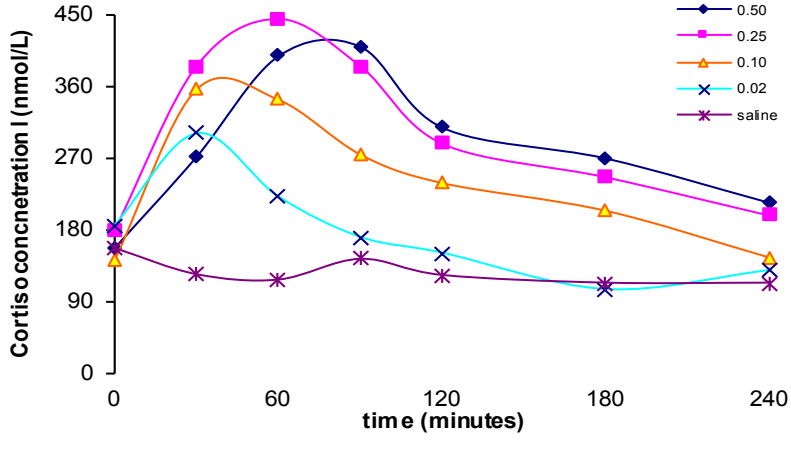

Figure 9a. Individual cortisol concentrations from one adult horses administered saline (0.9% NaCl) solution and each of four doses (0.02, 0.1, 0.25, and 0.5 µg/kg of cosyntropin.

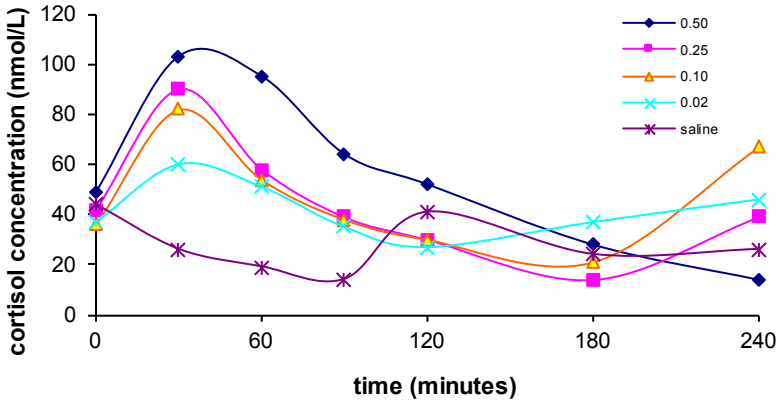

Figure 9b. Individual cortisol concentrations from one neonatal foal administered saline (0.9% NaCl) solution and each of four doses (0.02, 0.1, 0.25, and 0.5 µg/kg) of cosyntropin. Note the lower endogenous and post cosyntropin cortisol concentrations compared to the adult horse.

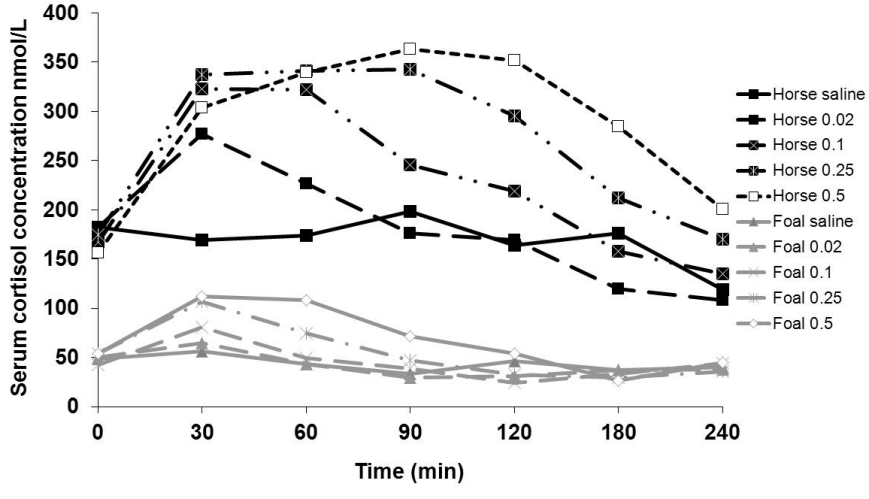

Figure 10. Mean serum cortisol concentrations in 11 healthy neonatal foals and 8 adult horses (mean ± SD age, 7.4 ± 3.5 years; range, 2 to 12 years) administered saline (0.9% NaCl) solution or 1 of 4 doses (0.02, 0.1, 0.25, and 0.5 µg/kg) of cosyntropin. Note the lower endogenous and post cosyntropin cortisol concentrations in the foals compared to the adult horses.

4.4 Project III: Insulin dysregulation in horses with systemic inflammatory response syndrome

4.4.1 Clinical data

Fifty-eight horses met the inclusion criteria of SIRS, ranging from 5 to 32 years of age. The gastrointestinal system was affected in 53 horses (91%), respiratory system in 3 (5%) and the neurologic and reproductive systems in 1 case each (2% each). Fifteen horses had an explorative laparotomy and, based on surgery or necropsy reports, 15 (28%) had an ischemic lesion.

Thirty-one horses (53%) had a SIRS score of 2, 18 horses (31%) of 3 and 9 horses (16%) of 4. There were 36 survivors (62%). A necropsy was performed in 16 non-survivors (73%).

4.4.2 Insulin

At admission, hyperinsulinemia (> 20 µIU/mL) was diagnosed in 21 horses (36%) and was not associated with survival (P = 0.21); however, serum insulin

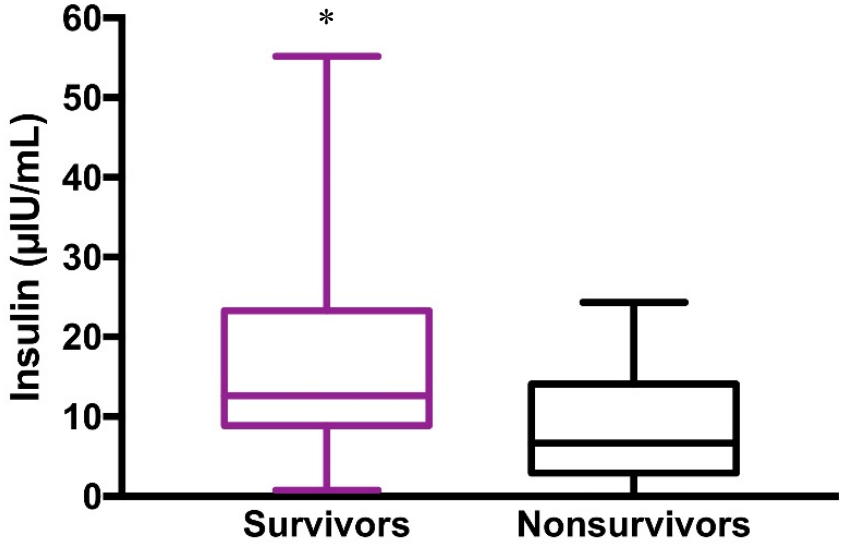

Figure 11. Serum insulin (µIU/mL) at admission in non-survivors and survivors (*P < 0.05).

was significantly lower in non-survivors (6.69 µIU/mL [0.06 – 24.34]) than survivors (12.6 µIU/mL [0.76 – 55.16]; P = 0.04, Figure 11).
Hyperinsulinemia at any time during hospitalisation (Day 0, 2, 4 or 6) was associated with survival (P = 0.02, Table 1 of paper III) with hyperinsulinemic horses 4 times more likely to survive. Severe hyperinsulinemia (insulin > 50 µIU/mL) was present in 6 horses but was not associated with survival (P = 0.39). Hyperinsulinemia or serum insulin concentration at any time during hospitalisation were not associated with SIRS group (P = 0.56 and P = 0.90 respectively).

The ROC curve showed that a serum insulin concentration > 8.82 µIU/mL (suggested optimal cut-off by the algorithm) was a poor diagnostic test to predict survival with an area under the ROC of 0.67 yielding to a sensitivity of 60.0% [36.1 – 80.9] and a specificity of 76.5% [58.8 – 89.3].

4.4.3 Glucose

At admission, hyperglycaemia (> 124 mg/dL) was diagnosed in 44 horses (88%) and was associated with non-survival (P = 0.03, Table 1 of paper III) with hyperglycaemic horses 5 times less likely to survive. At admission serum glucose was significantly higher in non-survivors (165 mg/dL [90 – 387] versus survivors 137 mg/dL [45 – 329], p = 0.04, Figure 12).

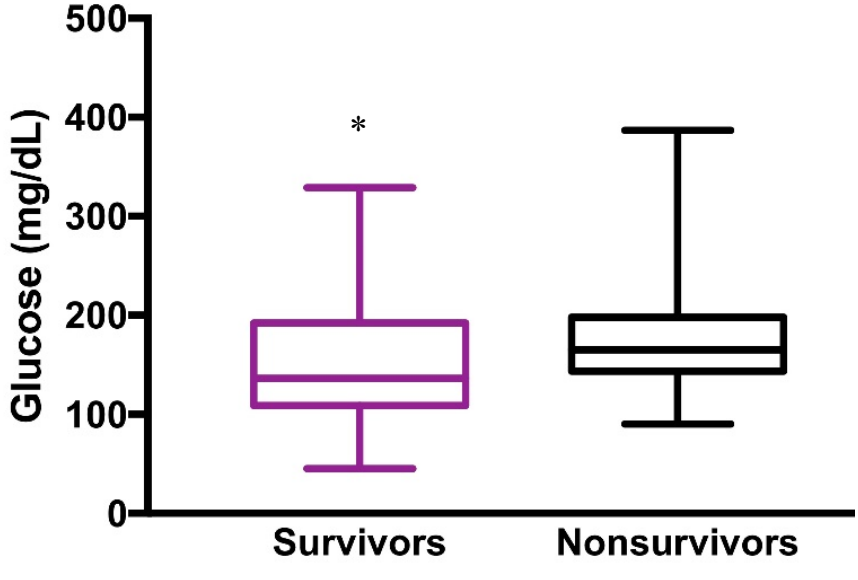

Figure 12. Figure 2: Serum glucose (mg/dL) at admission in survivors and non-survivors (*P < 0.05).

Presence of hyperglycaemia and serum glucose were both associated with SIRS group (P < 0.01) Figure 13).

Using a calculated optimal cut-off value of 150 mg/dL, the ROC curve showed that glucose was a poor predictor of survival with an area under the ROC curve of 0.66 resulting in a sensitivity of 71.4% [47.8 – 88.7] and a specificity of 63.9% [46.2 – 79.2].

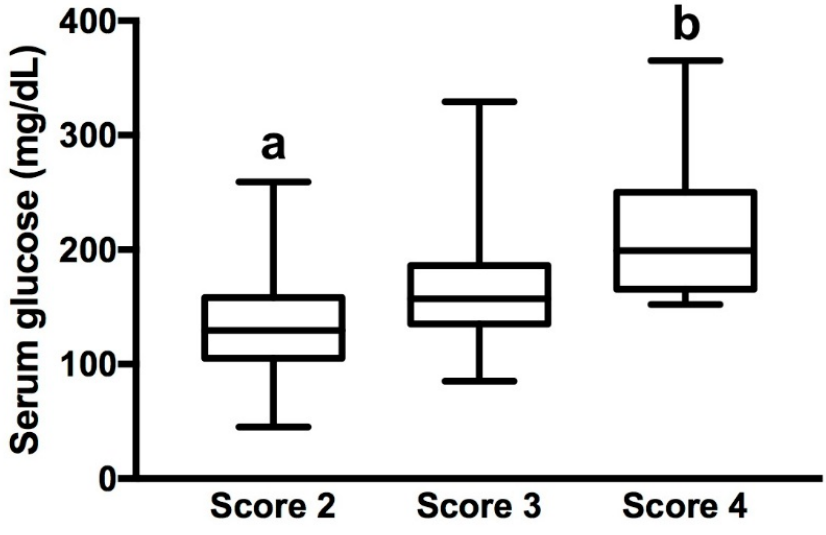

Figure 13. Serum glucose (mg/dL) at admission in horses with a SIRS score of 2, 3 and 4 (different letters indicate difference between groups, P < 0.05).

4.4.4 Ratio correlations

Insulin/glucose ratio, reflecting insulin secretion, was significantly lower in non-survivors (0.033 [< 0.01 – 0.22]) than survivors (0.1 [< 0.01 – 0.41]; P < 0.01, Figure 14). The ROC curve showed that an insulin/glucose value > 0.06 was a fair predictor of survival with an area under the ROC of 0.75 resulting in a sensitivity of 76.2% [52.8 – 91.8] and a specificity of 74.3% [56.7 – 87.5].

Glucose/insulin ratio, reflecting insulin sensitivity, was significantly higher in non-survivors (28.5 [1.8 – 709.1]) than survivors (10 [2 – 62.4]; P < 0.01, Figure15). A glucose/insulin ratio below 10, indicative of peripheral tissue insulin resistance, was obtained in 22 horses (38%) but was not associated with survival (P = 0.06) (Frank, 2011). A glucose/insulin ratio below 4.5, indicative of severe peripheral tissue insulin resistance, was obtained in 8 horses (14%) but

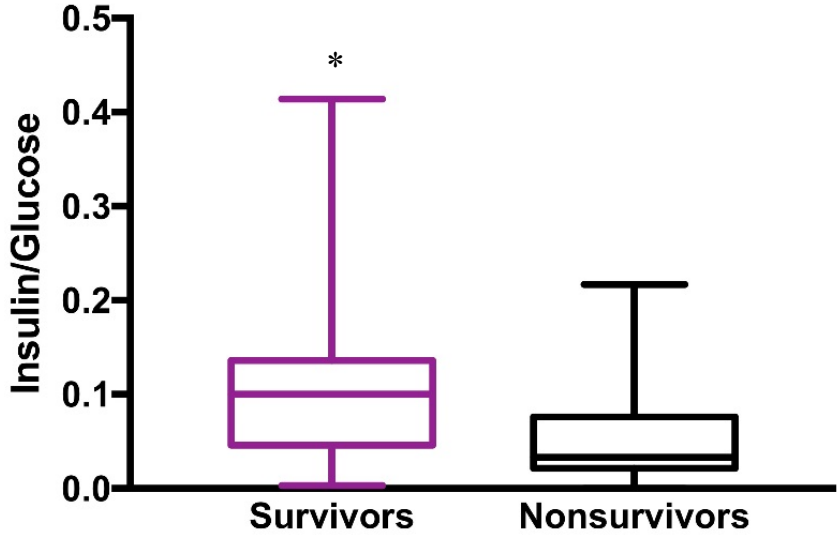

Figure 14. Insulin/glucose ratio, reflecting insulin secretion, in survivors and non-survivors (* P < 0.05).

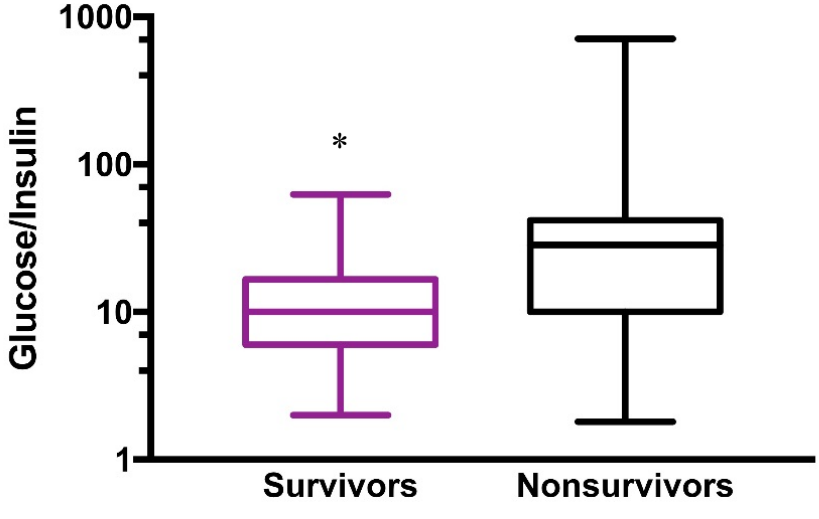

Figure 15. Glucose/insulin ratio, reflecting insulin sensitivity, in survivors and non-survivors (* P < 0.05).

was not associated with survival (p = 0.11).35 Using an optimal cut-off value of 12.5, the ROC curve showed that the glucose/insulin ratio was a fair diagnostic test to predict survival with an area under the ROC curve of 0.73 yielding a sensitivity of 71.4% [47.8 – 88.7] and specificity of 68.6% [50.7 – 83.2].

At admission, overall, there was no correlation between serum insulin and glucose (P = 0.13); however, in survivors, there was a significant correlation between serum insulin and glucose (P = 0.002, R2 = 0.25 Figure 16).

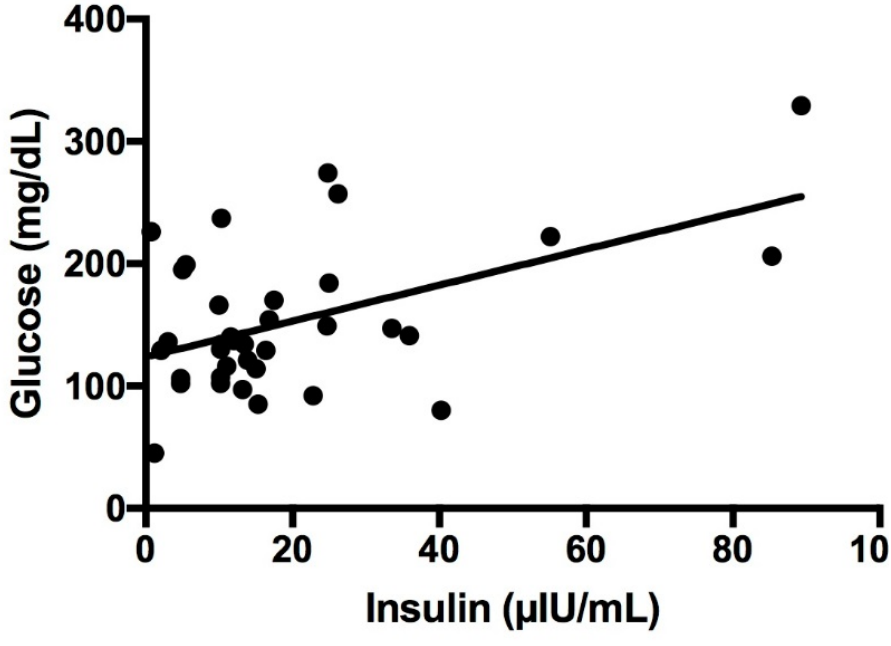

Figure 16. Correlation between serum insulin and glucose in survivors (p = 0.002, R^2 = 0.2468) (Bertin *et al.*, 2018a).

4.5 Project IV: Cortisol and ACTH concentrations in horses with systemic inflammatory response syndrome.

4.5.1 Clinical and outcome data

There were 151 horses ranging from 1 to 34 years of age (median of 11 years). Body systems affected were the gastrointestinal system in 144 horses (96.6%), the respiratory system in 3 horses (2%), and the neurologic and reproductive systems in 1 case each (0.7% each). An exploratory laparotomy was performed in 45 horses and, based on surgery (27 horses) or necropsy reports (23 horses), 50 horses had an ischemic lesion. There were 106 survivors. Of the 45 non-survivors, 30 cases were euthanized during the first evening, 4 of which had ruptured bowel at admission and 4 additional cases were euthanized during exploratory laparotomy. Of the 45 non-survivors, 2 horses were classed as SIRS score 0, 12 as score 1, 13 as score 2, 11 as score 3, and 7 as score 4; therefore 31 non-survivors (69%) met the criteria for SIRS. Of the 106 survivors, 17 horses were classed as SIRS score 0, 32 as score 1, 38 as score 2, 15 as score 3, 4 as score 4 and 1 horse as score 5; therefore, 58 horses (55%) met the criteria for SIRS. In non-survivors (n = 45) the median SIRS score: 2 [0 – 4] was higher than in survivors (n = 106): 2 [0 – 5]; P = .0041. Of the 50 horses with ischemic lesions, 18 survived and 32 did not survive (Figure 17).

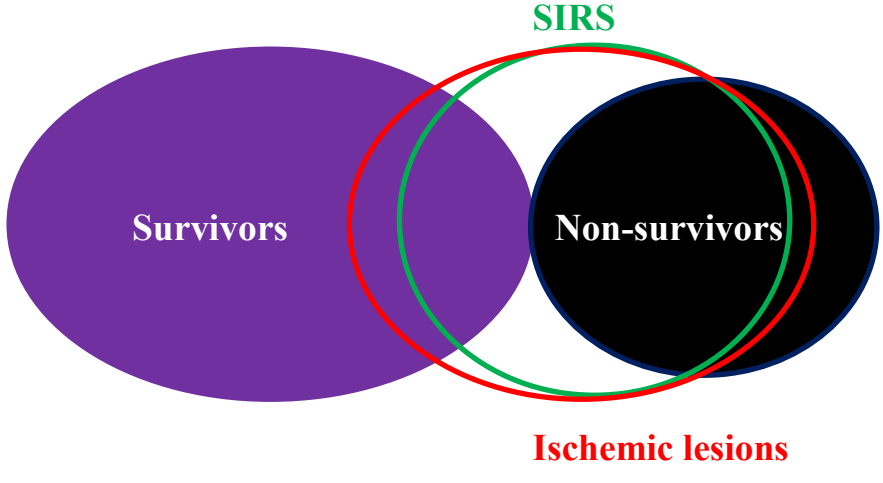

Figure 17. 106 survivors: 17 with SIRS and 18 with ischemic lesions
45 non-survivors: 31 with SIRS, 32 with ischemic lesions

4.5.2 Survival

ACTH

At admission, plasma ACTH was significantly lower in survivors (15.7 pg/mL [0.9 – 775.1]; n = 103) than non-survivors (56.7 pg/mL [5.8 – 563.8]; n = 45); P <.0001. In survivors, ACTH significantly decreased over time, P < 0.05 (Figure 18 and supplementary table 1 of paper IV). Of the surviving horses, (71/83, 85.6%) had an ACTH concentration within the reference interval at admission, whereas 49.2% of horses (32/65) with ACTH outside the reference interval survived. The number of horses with ACTH concentrations below reference interval for each day of hospitalisation is shown in Table 1 of paper IV. Of the surviving horses, (95/103) 92% had a normal ACTH concentration prior to discharge. When ACTH concentration was beyond the reference interval at admission, the OR for non-survival was 6.10 [2.73– 13.68], P < 0.0001. The ROC curve showed that an ACTH concentration > 30.5 pg/mL (the algorithm's suggested optimal cut-off) was a fair predictor of survival with an area under the ROC of 0.75, sensitivity of 71.1% [55.7 – 83.6] and specificity of 71.8% [62.1 – 80.3]. On day 2, 91.2% of horses (62/68) with ACTH within reference interval survived, whilst 68.6% of horses (11/16) with ACTH outside reference interval survived. For ACTH concentration outside the reference interval on day 2, the OR for non-survival was 4.70 [1.26 – 15.54], P = .016.

Cortisol

At admission, serum cortisol was significantly lower in survivors 9.7 µg/dL [1.5 – 38.6]; n = 104) than non-survivors (15.8 µg/dL [3.2 – 55.9]; n = 45): P =.0003. Overall cortisol decreased over time, P < 0.05 (Figure 18b). The ROC curve showed that cortisol > 11.7 µg/dL as a poor predictor of survival with an area under the ROC of 0.68, sensitivity of 68.9% [53.4 – 81.8], specificity of 67.3% [57.4 – 76.2] and a likelihood ratio of 2.1 for non-survival.

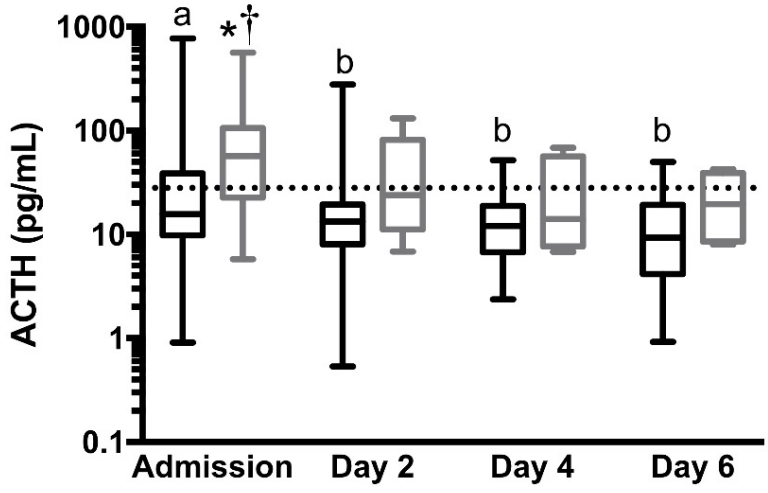

Figure 18a

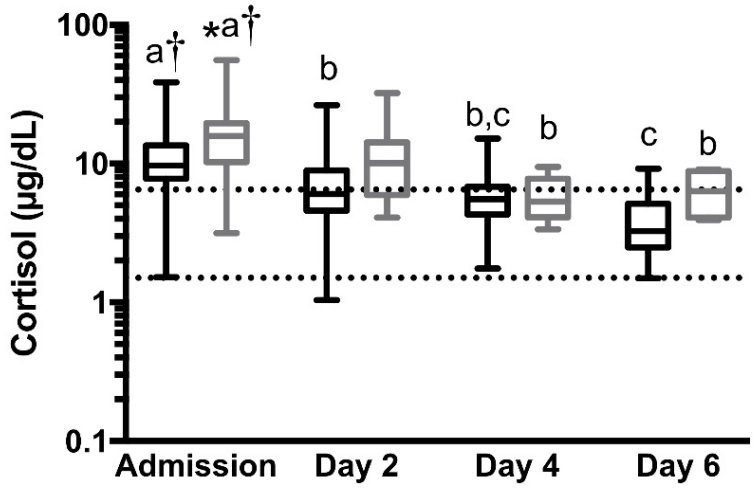

Figure 18b

*Figure 18*a-b. Plasma ACTH (1a), serum cortisol (1b) at admission, day 2, day 4 and day 6 of hospitalization in survivors (black) verses non-survivors (grey).

* difference between survivors and non-survivors at admission for ACTH ($P < .0001$), and cortisol ($P = .0003$). The reference range is marked by a dotted horizontal line.
† higher than upper limit of reference range for ACTH of 28.0 pg/mL ($P = .0001$), A different letter indicates a significant difference between days ($P < .05$).

ACTH/cortisol ratio

At admission, the ACTH/cortisol ratio was significantly higher in non-survivors (3.8 [0.8 – 32.6]; n = 45) than survivors (1.8 [0.1 – 66.6]; n = 101), P = 0.0001 (Figure 18c), but no changes were detected over time in survivors or non-survivors. The ROC curve showed that an ACTH/cortisol ratio concentration > 2.6 was a poor diagnostic test to predict survival with an area under the ROC of 0.696, with sensitivity of 64.4 [48.8 – 78.1], specificity of 67.3% 67.3 [57.3 – 76.3] and a likelihood ratio of 1.97 for non-survival.

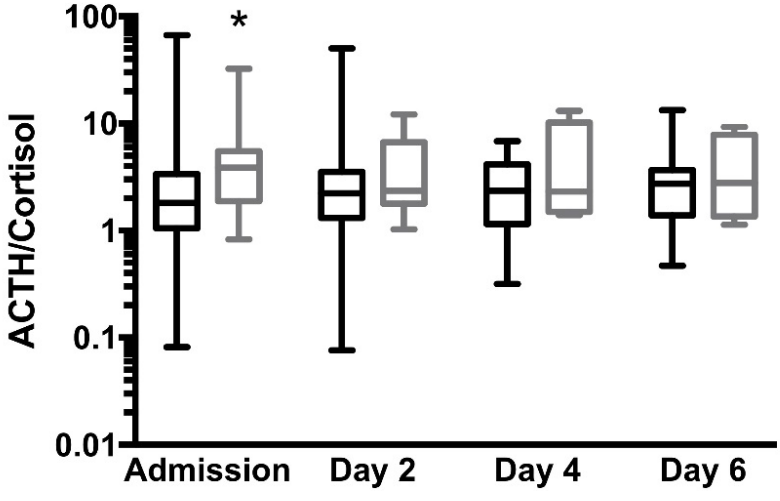

*Figure 18*c. ACTH/cortisol ratio at admission, day 2, day 4 and day 6 of hospitalization in survivors (black) verses non-survivors (grey).

4.5.3 SIRS score

ACTH

At admission, horses with SIRS had higher ACTH concentrations at admission compared with days 4 and 6 (P < .05), but there was no difference was detected between horses with and without SIRS (P = .19) (Figure 19a and supplementary table 2 of paper IV) No statistically significant difference in ACTH concentrations were detected between any individual SIRS score groups (P = 0.27) at admission, or over time for horses with SIRS score of 0, 1, 2 or 3..

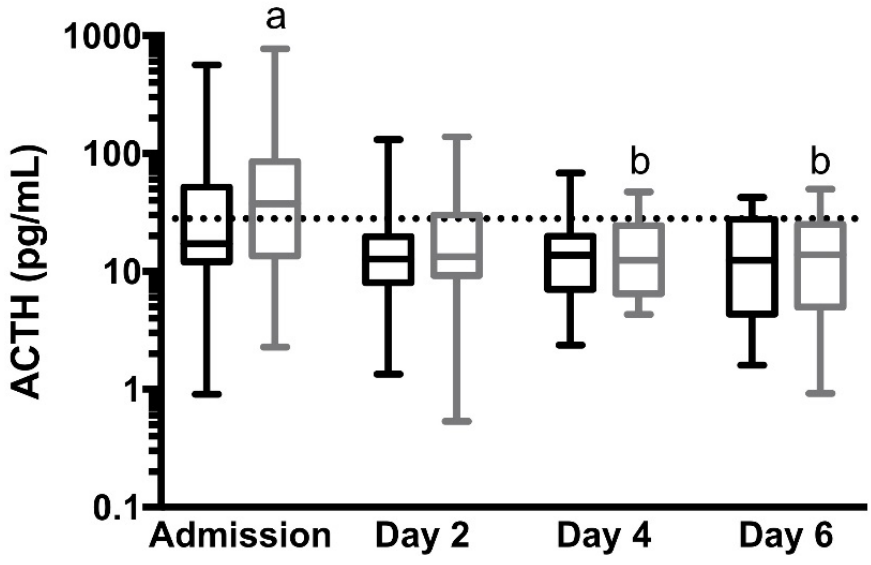

Figure 19a.

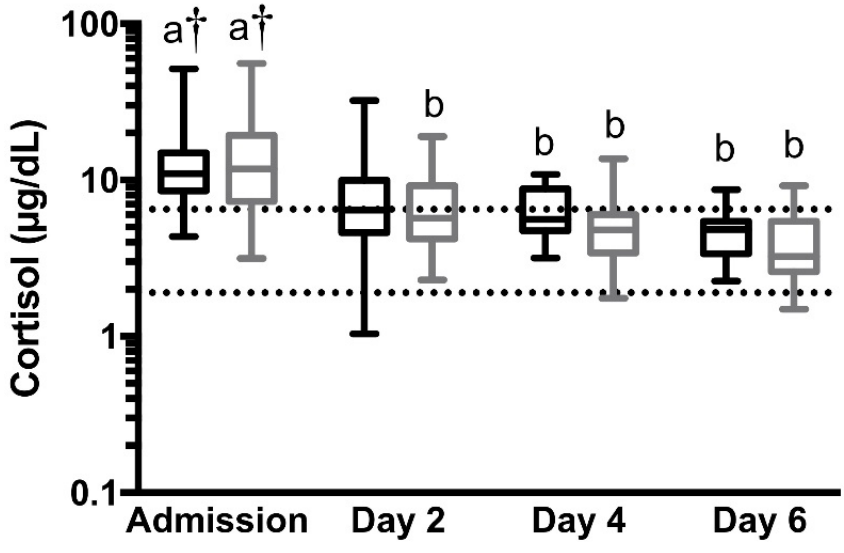

Figure 19b.

Figure 19a-b. Plasma ACTH (19a) and serum cortisol (19b) at admission, day 2, day 4 and day 6 of hospitalization for horses with SIRS (grey) verses without SIRS (black).

The reference range is marked by a dotted horizontal line.

† higher than upper limit of reference range for cortisol 6.5 µg/dL ($P < .05$). A different letter indicates a significant difference between days ($P < .05$)

However, the ACTH concentration was higher than the reference range at admission and higher than day 4 for horses with a SIRS scores cores of 4 and 5 ($P < .05$) (supplementary Figure 1a and supplementary table 3 of paper IV)

Cortisol

At admission, horses with SIRS had higher cortisol concentrations at admission compared with days 2, 4 and 6 ($P < .05$), while horses without SIRS had higher cortisol at admission compared with days 2 and 4 ($P < .05$). At admission Both SIRS and no SIRS groups of horses had cortisol concentrations higher than the reference range at admission (Figure 19b). The cortisol concentration was above reference range at admission in all SIRS groups ($P < .05$), and there were no differences between SIRS groups ($P = 0.21$) and no difference between horses with or without SIRS ($P = .88$) at admission.). The cortisol concentration was higher at admission compared to day 2 and 4 in the SIRS score 1, 2 and 3 groups ($P < .05$) (supplementary Figure 1b of paper IV).

ACTH/cortisol ratio

Horses with SIRS had higher ACTH/cortisol ratio at admission compared to horses without SIRS ($P = .042$). (Figure 19c) There were no differences detected in the ACTH/cortisol ratio between SIRS score groups ($P = 0.21$ at admission) or over time. (supplementary Figure 1c of paper IV)

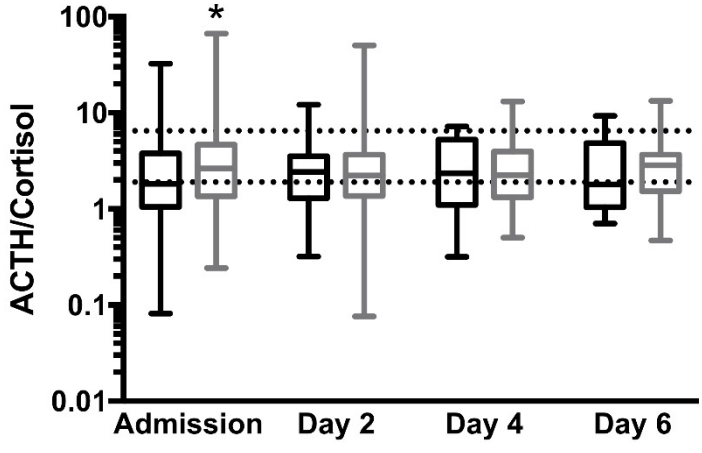

Figure 19c. ACTH/cortisol ratio at admission, day 2, day 4 and day 6 of hospitalization for horses with SIRS (grey) verses without SIRS (black).

* difference between non-SIRS and SIRS at admission for ACTH/cortisol ratio ($P = .04$).

4.5.4 Ischemic gastrointestinal lesion

Of the 50 horses with ischaemic lesions only 18 (36%) survived, while 87.1% (88/101) (87.1%) with non-ischaemic lesions survived. Horses with an ischemic lesion were 12 times more likely to not to survive, (OR: 12.03 [5.36 – 25.54], P < 0.0001). Although not statistically significant, there was a trend for the SIRS score in horses with ischemic lesions (n = 51: 2 [0 – 5]) to be higher than those with non-ischemic lesions (n = 100: 2 [0 – 4]: P = 0.076).

ACTH

At admission horses with ischemic gastrointestinal lesions had higher plasma ACTH concentrations than those without ischemic lesions at admission (P < .0001). Horses at admission horses with ischemic lesions had ACTH concentrations greater than the reference range at admission interval (P < .05), and these declined by days 2, 4 and 6 (P < .05). There was no change over time for horses with non-ischemic lesions. (Figure 20a and supplementary table 4 of paper IV). The ROC curve showed that an ACTH concentration > 28.5 pg/mL was a poor diagnostic test to predict ischemia with an area under the ROC of 0.73, with sensitivity of 66.7% [52.1 – 79.2], specificity of 69.1% [58.9 – 78.1] and a likelihood ratio of 2.16 for presence of an ischemic gastrointestinal lesion.

Cortisol

At admission, horses with ischemic lesions had higher cortisol concentrations than those without ischemic lesions at admission (P < .0001). Horses with and without ischemic lesions had higher cortisol concentrations at admission which declined throughout hospitalisation over time (P < .05). Cortisol was higher than the reference range at admission and on day 2 for those horses with ischemic lesions and at admission for those with non-ischemic lesions (P < .05) (Figure 20b) of paper IV). The ROC curve showed that a cortisol concentration > 11.6 µg/dL was a poor predictor of ischemia with an area under the ROC of 0.73, with sensitivity of 70.6% [56.2 – 82.5], specificity of 69.4% [59.3 – 78.3] and a likelihood ratio of 2.31) for presence of an ischemic gastrointestinal lesion.

ACTH/cortisol ratio

At admission horses with ischemic lesions had higher ACTH/cortisol ratio than those without ischemic lesions at admission (P = .0069), but there was no change in either group over time. (Figure 20c of paper IV). The ROC curve showed that an ACTH/cortisol ratio > 2.4 was a poor predictor ischemia with an area under the ROC of 0.63, with sensitivity of 62.8% [48.1 – 75.9], specificity of 64.2% [53.7 – 73.8] and a likelihood ratio of 1.75) for presence of an gastrointestinal ischemic lesion.

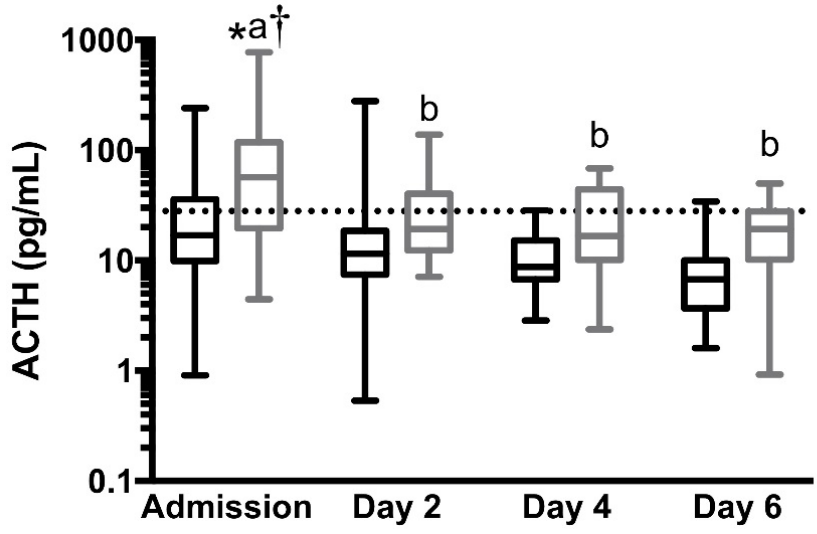

Figure 20a.

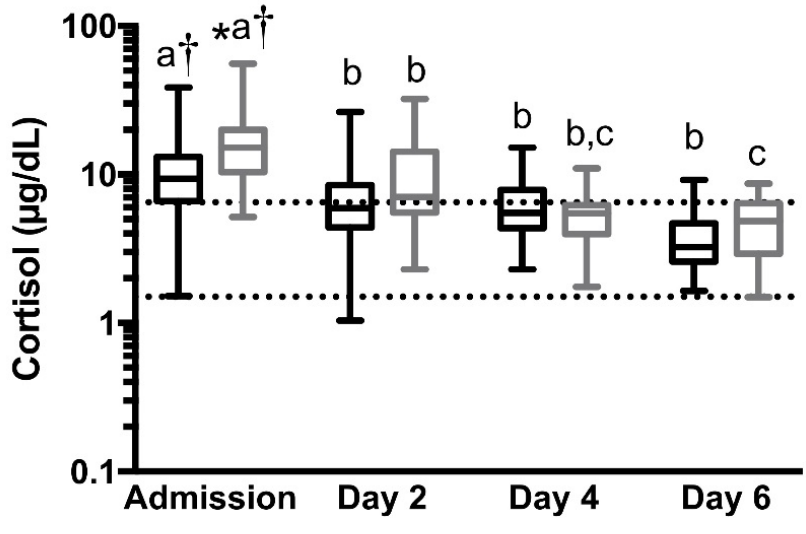

Figure 20b.

Figure 20a-b. Plasma ACTH (20a) and serum cortisol (20b) at admission, day 2, day 4 and day 6 of hospitalization in horses with ischemic gastrointestinal lesions (grey) verses horses without ischemic lesions (black).

* difference between ischemic and non-ischemic at admission for ACTH ($P < .0001$) and cortisol ($P < .0001$).
The reference range is marked by a dotted horizontal line.
† higher than upper limit of reference range for ACTH of 28.0 pg/mL and cortisol: 6.5 µg/dL ($P < .05$). A different letter indicates a significant difference between days ($P < .05$).

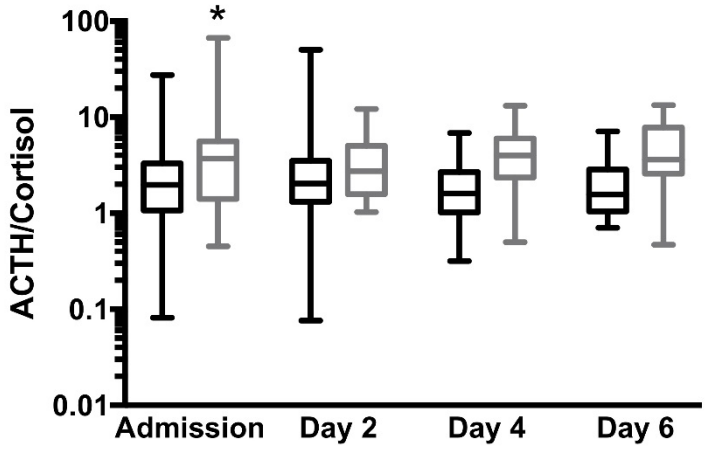

Figure 20c. ACTH/cortisol ratio at admission, day 2, day 4 and day 6 of hospitalization in horses with ischemic gastrointestinal lesions (grey) verses horses without ischemic lesions (black).
* difference between ischemic and non-ischemic at admission (P = .0069).

4.6 Correlations between endocrine parameters in sick horses

4.6.1 ACTH, Cortisol, insulin and glucose correlations in horses

Overall, glucose and insulin (Figure 16), glucose and ACTH, and ACTH and cortisol were positively correlated (n = 126, P = 0.027, n = 141, P = 0.051 and n= 141, P = 0.02, respectively). In horses with SIRS, only cortisol and glucose were positively correlated (n = 68, P = 0.003 Figure 21a). In horses without SIRS, only glucose and insulin were positively correlated (n = 63, P = 0.005, Figure 21b). In survivors, glucose and insulin were positively correlated (n = 87, P = 0.0008, Figure 21c), with a trend towards a positive correlation between glucose and ACTH (n =102, p = 0.058). In non-survivors, no correlations were observed between parameters (Stewart *et al.*, 2018).

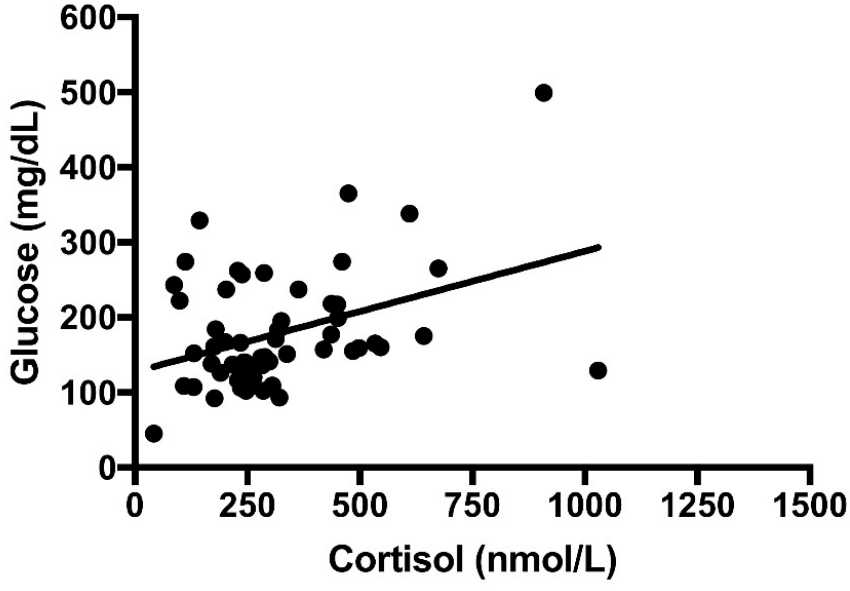

Figure 21a. In horses with SIRS, only cortisol and glucose were positively correlated (n = 68, P = 0.003

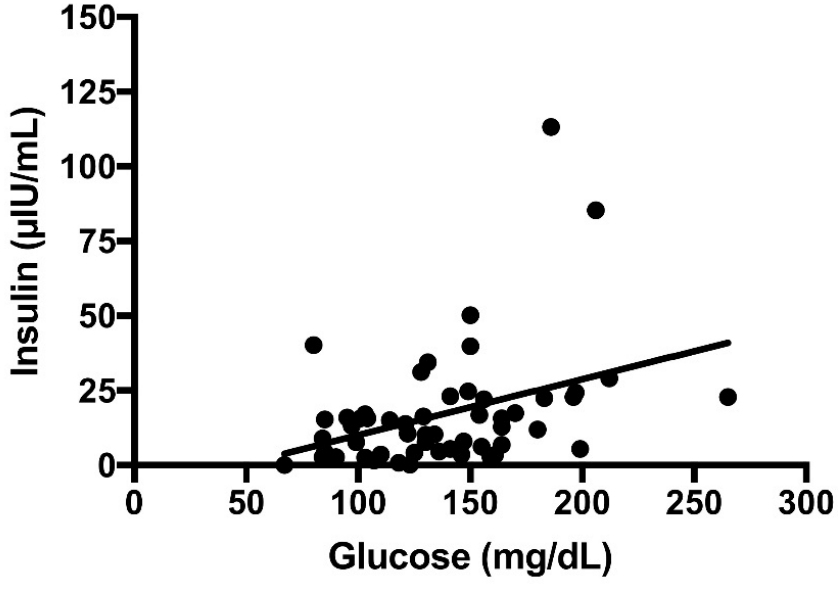

Figure 21b In horses without SIRS, only glucose and insulin were positively correlated (n = 63, P = 0.005).

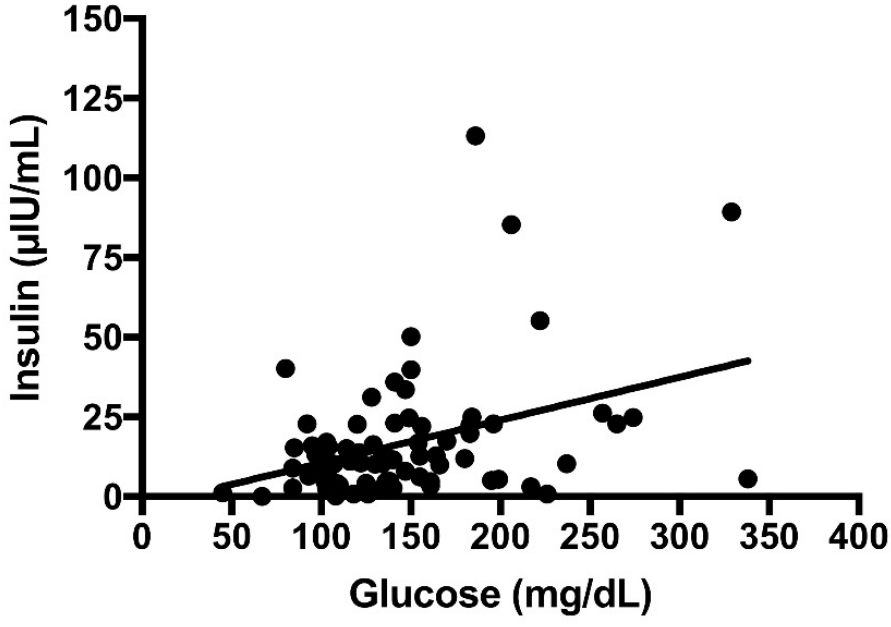

Figure 21c In survivors, only glucose and insulin were positively correlated (n = 87, P = 0.0008).

4.7 Project V: Identification of Adrenal Dysfunction in Critically Ill Adult Horses.

4.7.1 Clinical and outcome data

There were 58 horses were included, ranging in age from 1 to 24 years of age (median of 10 years). There were 57 horses (98%) with a final diagnosis involving the gastrointestinal system. There were 29 medical cases: 7 with colitis [3 *Aeromonas* spp (1 with haemoabdomen and a large colon impaction), 2 Salmonella spp, 2 of undetermined aetiology]; 6 ileal impactions, 5 undiagnosed medical colics, 3 impactions of the small colon, 2 impactions of the large colon, 2 left dorsal displacements and 1 each of anterior enteritis, rectal mass, sand colic, oesophageal squamous cell carcinoma and. There were 23 surgically managed cases: 8 right dorsal displacements (1 ruptured at surgery and one euthanized on day 3 due to mesenteric melanomas observed at surgery), 4 strangulating lipomas, 4 torsions of the large colon, 3 small intestinal entrapments in the epiploic foramen, 1 each of small colon impaction, idiopathic peritonitis, focal jejunal eosinophilic enteritis and incarcerated small intestine due to a *Streptococcus equi* subsp. *equi* abscess that was euthanized at surgery. Five cases were euthanized at presentation due to poor prognosis: 3 with strangulating small intestinal lesions and 1 case each of torsion of the large colon and severe gastritis. There were 14 cases with an ischemic gastrointestinal lesion. There was also 1 horse with severe pleuropneumonia.

There were 44 (76%) survivors. Among the 14 (24%) non-survivors, 5 cases were euthanized at admission, 2 during exploratory laparotomy and 1 case of *Salmonella* spp colitis and septicaemia was euthanized during the first evening. Two more cases were euthanized prior to day 4 and 1 prior to day 6 sampling. Of the non-survivors, ACTH and cortisol results were available at admission for 14 cases, on day 2 for 6 cases, on day 4 for 4 cases and on day 6 for only 3 cases. For the survivors, ACTH and cortisol results were available at admission for 44 cases, on day 2 for 40 cases, on day 4 for 31 cases and on day 6 for 19 cases. Of the 14 non-survivors, 5 horses were classified as SIRS score 1, 4 horses as score 2, 3 horses as score 3, 2 horses as score 4; therefore 9 horses (64%) met the criteria for SIRS. Of the 44 survivors, 6 horses were classified as SIRS score 0, 10 horses as score 1, 19 horses as score 2, 9 horses as score 3, therefore 28 horses (64%) with SIRS. Overall, 37 horses (64%) met the criteria for SIRS.

4.7.2 Outcome of survival

The median SIRS score in non-survivors (2 [1 – 4]; n = 14) was not significantly higher than in survivors: (2 [0 – 3]; n = 44; P = 0.30) Plasma ACTH was significantly higher in non-survivors (37.5 pg/mL [8.4 – 134.8]; n = 14) than survivors (16.5 pg/mL [2.0 – 775.1]; n = 44:P = 0.006) at admission and also on day 2, with non-survivors higher (54.8 pg/mL [6.8 – 131.6]; n = 6) than survivors (13.6 pg/mL [0.5 – 278.0]; n = 40: P = 0.006). Overall, ACTH significantly decreased over time P = 0.009 when all horses were considered (n = 58). (Figure 22a)

Serum cortisol at baseline was significantly higher in non-survivors (15.1 µg/dL [3.9 – 51.3]; n = 14) than survivors (9.4 µg/dL [3.9 – 38.6]; n = 44: P = 0.02) at admission. In all horses, cortisol decreased over time, P < 0.0001 (Figure 22b). After administration of cosyntropin, there was no difference in cortisol at T = 30 between non-survivors and survivors (P = 0.14), or over time (P = 0.30). There was no difference in delta-cortisol between non-survivors and survivors (P = 0.75), or over time (P = 0.87).

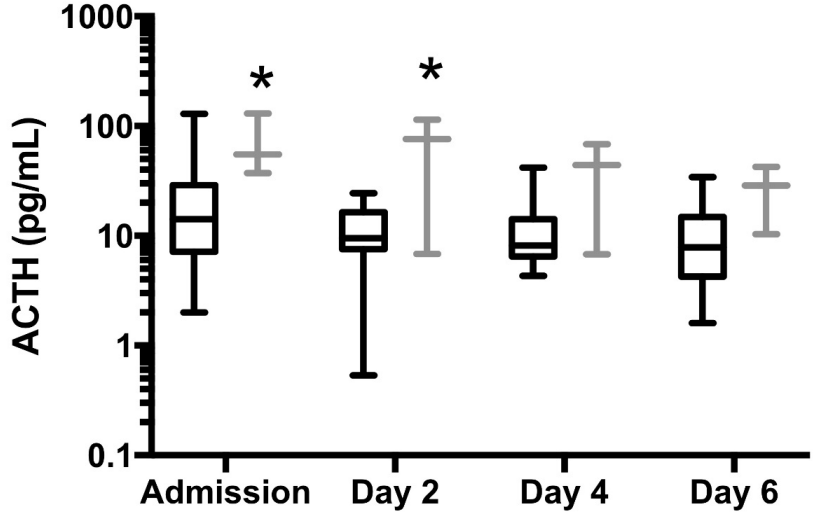

Figure 22a. Plasma ACTH at admission, day 2, day 4 and day 6 of hospitalization in survivors (black) verses non-survivors (grey).

* difference between survivors and non-survivors at admission (P = 0.036) and day 2 and (P = 0.0060).

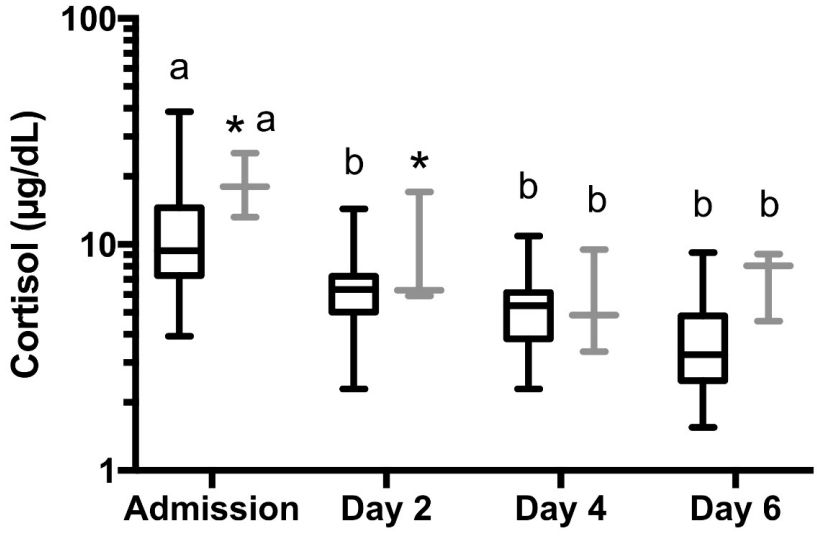

Figure 22b. Serum cortisol at admission, day 2, day 4 and day 6 of hospitalization in survivors (black) verses non-survivors (grey).

* difference between survivors and non-survivors at admission (P = 0.021).
A different letter indicates a significant difference between days (P < .05)

For ACTH stimulation, there was no significant effect of stimulation (P = 0.17), however there was a difference between basal cortisol between survivors and non-survivors (P = 0.02), but not at T = 30 minutes (P = 0.64). (Figure 23)

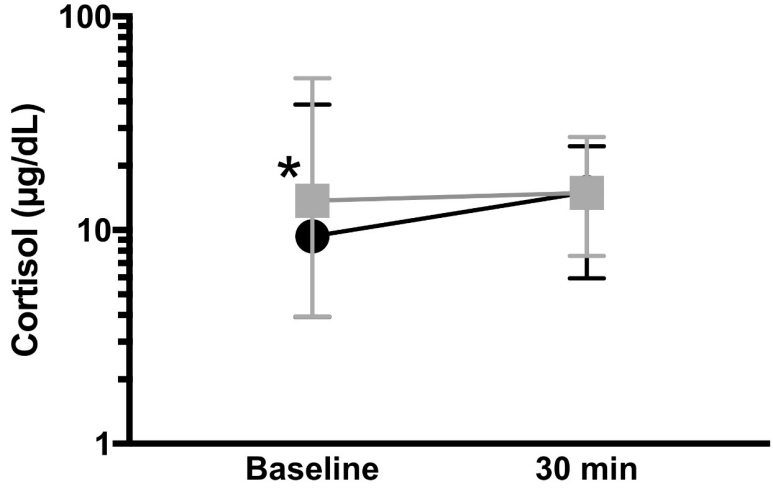

Figure 23. Mean ± SD serum cortisol at baseline and 30 minutes after administration of 0.1 µg/kg IV of cosyntropin at admission for survivors (black) verses non-survivors (grey).

* difference basal cortisol between survivors and non-survivors (*P* = 0.018). There was no difference at 30 minutes (*P* = 0.64).

4.7.3 Outcome of SIRS score

There was no difference in the ACTH, basal cortisol, or delta-cortisol concentrations at admission in horses with or without SIRS (P = 0.73, 0.27, 0.5), however the T =30 minutes cortisol concentration was lower in horses with SIRS (14.8 ± 4.4 µg/dL) verses versus those without SIRS (17.8 ± 5.0 µg/dL; P = 0.002). Overall, ACTH, basal cortisol (Figure 24) and T = 30 cortisol but not delta-cortisol decreased over time (P = 0.01, < 0.0001, 0.06 and 0.96). There was a significant effect of ACTH stimulation (P = 0.05), and a difference between horses with and without SIRS (P = 0.025) due to an effect at T = 30 minutes (P = 0.05) but not basal cortisol (P = 0.32). There was an overall significant effect of ACTH stimulation (P = 0.05) and an effect of SIRS (P = 0.02), where at T = 30 minutes the cortisol concentration was higher in horses without SIRS (P = 0.05).

At admission, no statistically significant differences were detected in ACTH, basal cortisol or delta-cortisol concentrations between any individual SIRS score groups (P = 0.47, 0.22, 0.82), however, the T = 30 cortisol was lower between horses with SIRS score 2 (12.4 µg/dL [5.9 – 18.3]) than SIRS score 1 (18.1 µg/dL [12.2 – 27.3]; P = 0.002). There was an overall effect of ACTH stimulation (P = 0.03), and effect of SIRS score (P = 0.05).

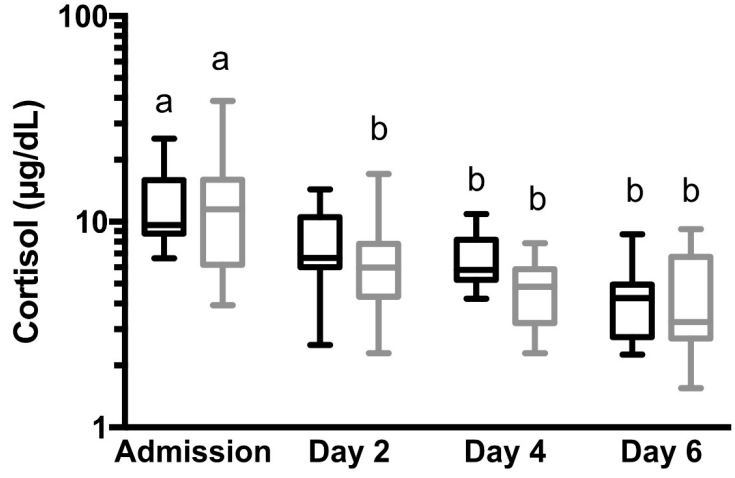

Figure 24. Serum cortisol at admission, day 2, day 4 and day 6 of hospitalization for horses with SIRS (grey) verses without SIRS (black).

A different letter indicates a significant difference between days ($P < .05$)

4.7.4 Outcome of ischemic gastrointestinal lesion

Of the 19 horses with ischaemic lesions only 53% (10/19) survived, while 87% (34/39) with non-ischaemic lesions survived. At admission, basal cortisol concentrations were higher in horses with ischemic lesions (13.2 µg/dL [6.9 – 51.3]) than those without ischemic lesions (9.4 µg/dL [3.9 – 38.6]; P = 0.04). There were no differences detected between horses with and without ischemic lesions at admission for ACTH, T =30 cortisol, or delta-cortisol (P = 0.18, 0.22, 0.29). ACTH was higher in horses with non-ischemic lesions at admission verses day 2 (P = 0.04), day 4 (P = 0.04) and day 6 (0.04). (Figure 25a) In horses with ischemic lesions, the basal cortisol was higher at admission versus day 4 (P = 0.003) and day 6 (P = 0.0007). For horses with non-ischemic lesions basal

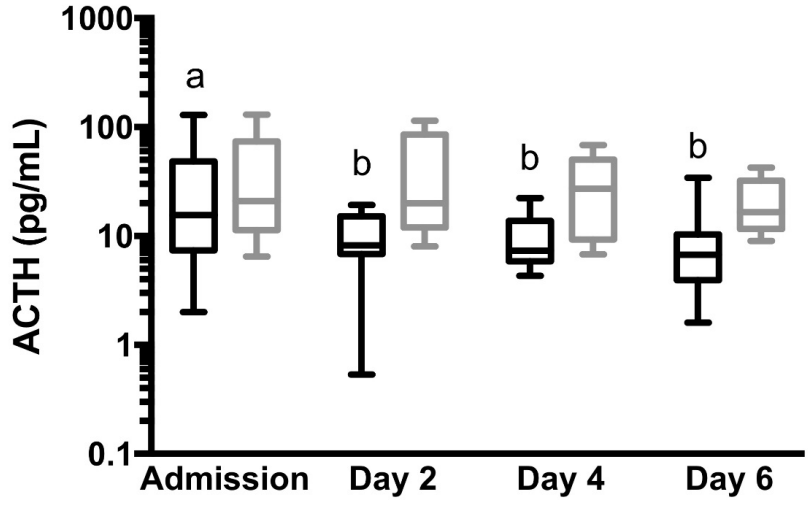

Figure 25a.

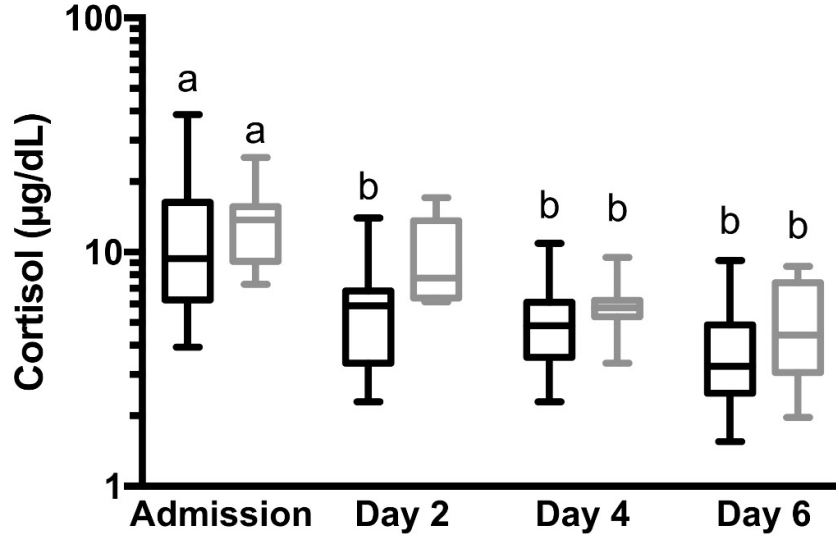

Figure 25b.

Figure 25a-b. Plasma ACTH (25a) and serum cortisol (25b) at admission, day 2, day 4 and day 6 of hospitalization in horses with ischemic gastrointestinal lesions (grey) verses horses without ischemic lesions (black).

** difference between ischemic and non-ischemic at admission for ACTH (P = 0.18) and cortisol (P = 0.036). A different letter indicates a significant difference between days (P < .05)..*

cortisol was higher at admission verses day 2 (P = 0.001), day 4 (P = 0.0003) and day 6 (P <0.0001). (Figure 25b). T = 30 cortisol was higher between admission and day 6 (P = 0.031) for horses with non-ischemic lesions.
There was a significant effect of ACTH stimulation (P = 0.005) due to an increase in cortisol concentrations in horses without ischemic lesions (P = 0.005), but no difference in horses with ischemic lesions (P = 0.97). There was also an effect of ischemia (P = 0.04), due to a higher basal cortisol concentration at in horses with ischemic lesions (P = 0.02) compared to horses without ischemic lesions, however there was no difference at T = 30 minutes (P = 0.59). (Figure 26)

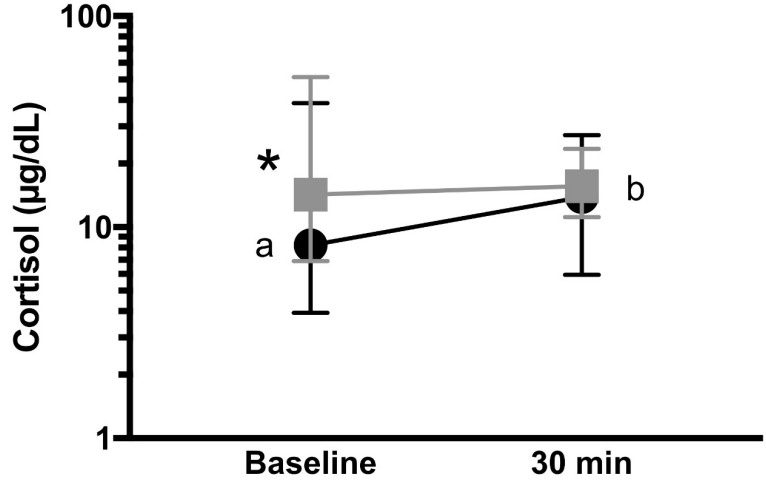

Figure 26. Mean ± SD serum cortisol at baseline and 30 minutes after administration of 0.1 µg/kg IV of cosyntropin at admission, for horses with ischemic gastrointestinal lesions (grey) and those without ischemic lesions (black).

* difference between basal cortisol in horses with ischemic and non-ischemic lesions (P = 0.018). There was no difference at 30 minutes (P = 0.59). Different letters indicate a difference between basal cortisol concentrations and 30 minutes post cosyntropin in horses with non-ischemic lesions (P = 0.0047). There was no difference between basal and 30 minutes cortisol concentrations for horses with ischemic lesions (P = 0.97).

4.7.5 Adrenal dysfunction

An inappropriately low basal cortisol less than the proposed cut-off of < 9.7 µg/dL, was detected in 35/58 (55%) of horses at some time during hospitalisation; 26/58 (45%) at admission, 37/48 (77%) on day 2, 32/35 (91%) on day 4 and 22/22 (100%) on day 6. An inappropriately low basal cortisol than

the proposed cut-off of < 4.51 µg/dL was identified in 22/58 (38%) of horses at some time during hospitalisation; 4/58 (7%) at admission, 5/48 (10%) on day 2, 10/35 (29%) on day 4 and 14/22 (64%) on day 6.

An inappropriately low delta-cortisol < 5.65 µg/dL was identified in 47/58 (81%) of horses; 32/52 (61%) at admission, 24/42 (57%) on day 2, 12/30 (40%) on day 4 and 6/18 (33%) on day 6. An inappropriately low delta-cortisol < 4.15 µg/dL was identified in 39/58 (67%) of horses; 26/52 horses (50%) at admission, 11/42 (26%) on day 2, 7/30 (23%) on day 4 and 4/18 (22%) on day 6. (Table 1 of paper V) Twelve critically ill horses were diagnosed with RAI/CIRCI (Table 2 of paper V).

4.8 Adrenal gland histopathology

Adrenal gland autolysis occurs rapidly and necropsies typically did not occur until 12-36 hours after death, therefore no useful correlations could be made between the presence of adrenal gland hemorrhage and necrosis and endocrinological test results.

The following 3 cases are presented:

4.8.1 Case number: 1091166

A 3 year old Tennessee Walking horse gelding presented for colic with a heart rate of 60 beats per minute and was estimated to be 8% dehydrated. The horse had a SIRS score = 2. He had 8 litres of nasogastric reflux at admission. Serosanguinous abdominal fluid was obtained by abdominocentesis, which had a with a normal white blood cell count of 1,600 cells /µL. Rectal palpation identified desiccated feed in large intestine and multiple loops of distended small intestine. The admission ACTH = 233 pg/mL, cortisol = 55.8 µg/dL (the highest cortisol of any patient) and ACTH/cortisol ratio = 4.17. Due to the guarded prognosis and limited finances the horse was euthanized and strangulated small intestine from a small intestinal volvulus was diagnosed at post mortem.

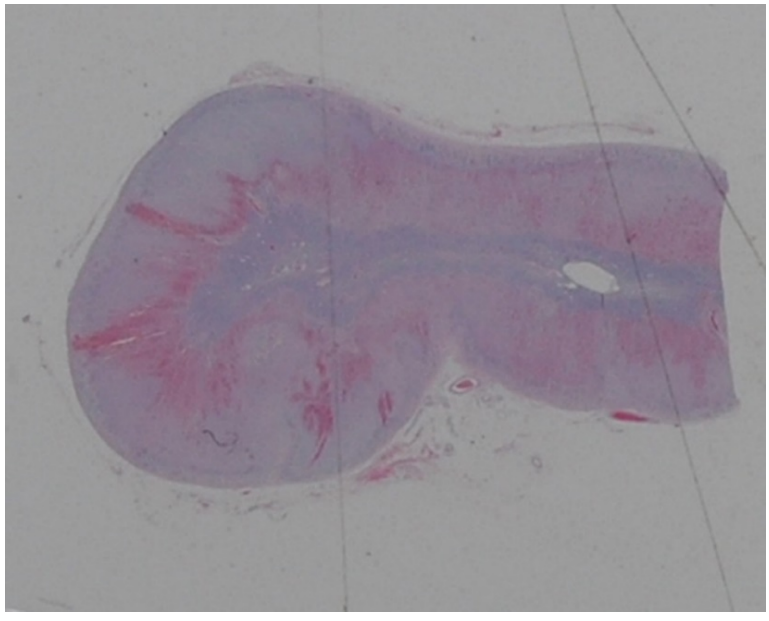

Figure 27a Gross image. There is congestion with blood visible in the vessels radiating outward towards the capsule through the *zona reticularis* and *glomerulosa*. Image courtesy of Dr. Kelly Joiner DVM, PhD, DACVP.

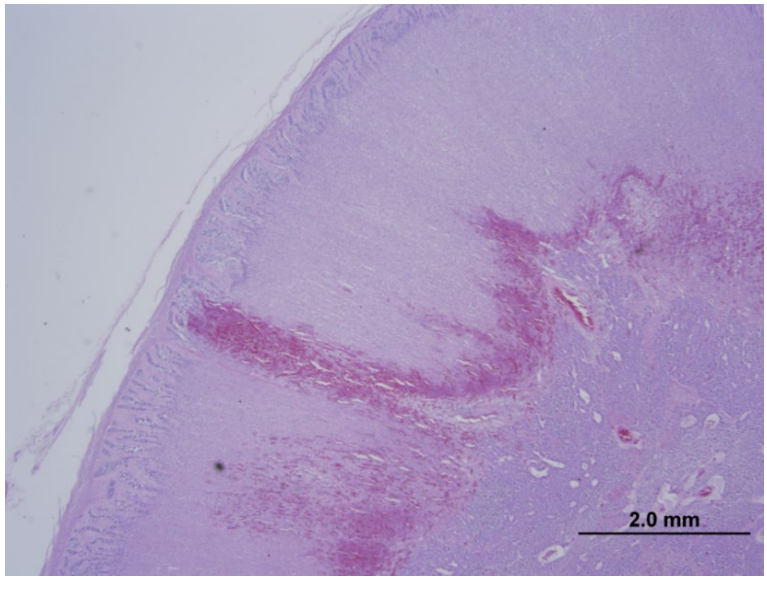

Figure 27b. Sub-gross histopathologic image of hemorrhage along the corticomedullary junction. There is congestion with blood visible in the vessels radiating outward towards the capsule through the *zona reticularis* and *glomerulosa*. Image courtesy of Dr. Kelly Joiner DVM, PhD, DACVP

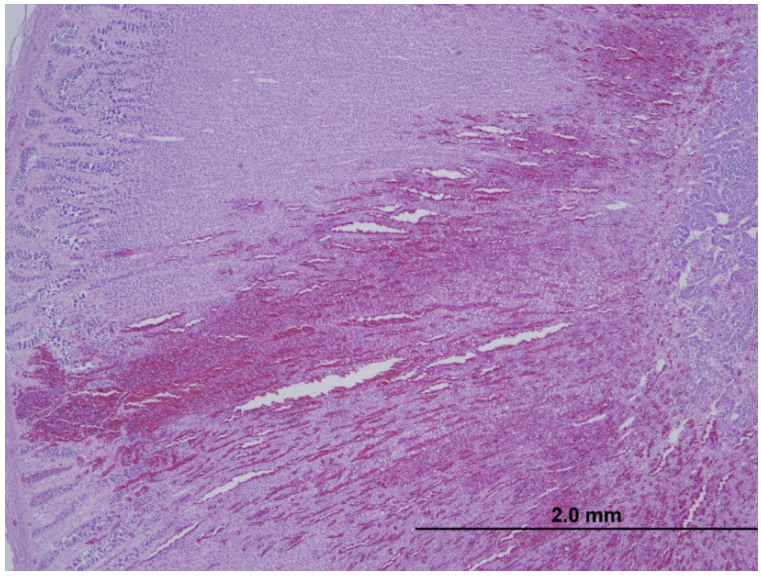

Figure 27c. Corticomedullary hemorrhage- enlargement. Image courtesy of Dr. Kelly Joiner DVM, PhD, DACVP.

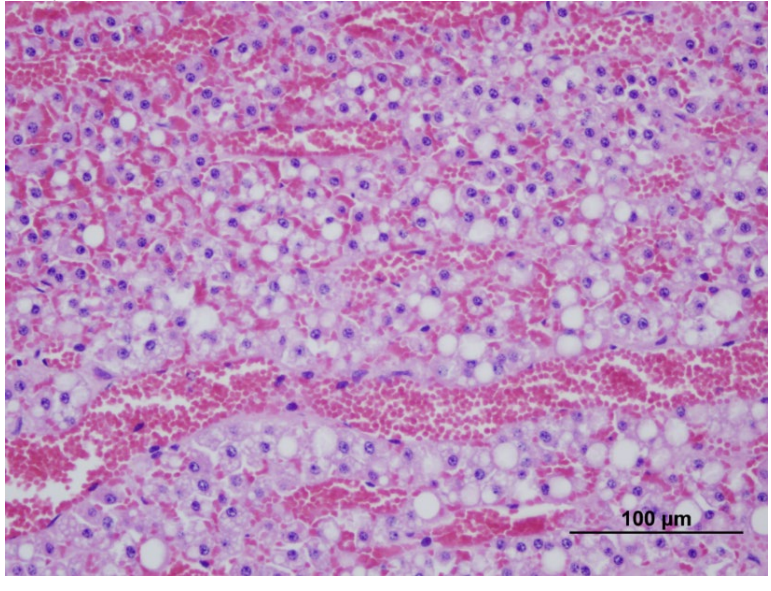

Figure 27d Vascular congestion of the corticomedullary region. Vacuolar change of the cells of the *zona reticularis* with lipid droplets and/ or glycogen secondary to the stress response. Cells exhibiting this could theoretically be saved/repaired but this indicates that they have started down a pathway that ultimately ends in apoptosis (cell death/atrophy/necrosis). Image courtesy of Dr. Kelly Joiner DVM, PhD, DACVP.

4.8.2 Case number: 1091166

A 14 year old draft horse gelding that presented for colic and had a soft mass palpable in the right upper quadrant rectally. The gelding was treated medically for ceacal impaction for 4 days then an exploratory laparotomy was performed on day 5. A typhlotomy was performed but the cecum ruptured at its base during surgery. The horse had a SIRS score of 1 at admission, and cortisol and ACTH were only measured at admission (ACTH = 7.6 pg/mL, cortisol = 5.17 µg/dL, ACTH/cortisol ratio = 1.47. The horse was not particularly sick over the course of hospitalisation with heart rate 56 beats per minute at admission, and the heart rate never exceeded 41 beats per minute

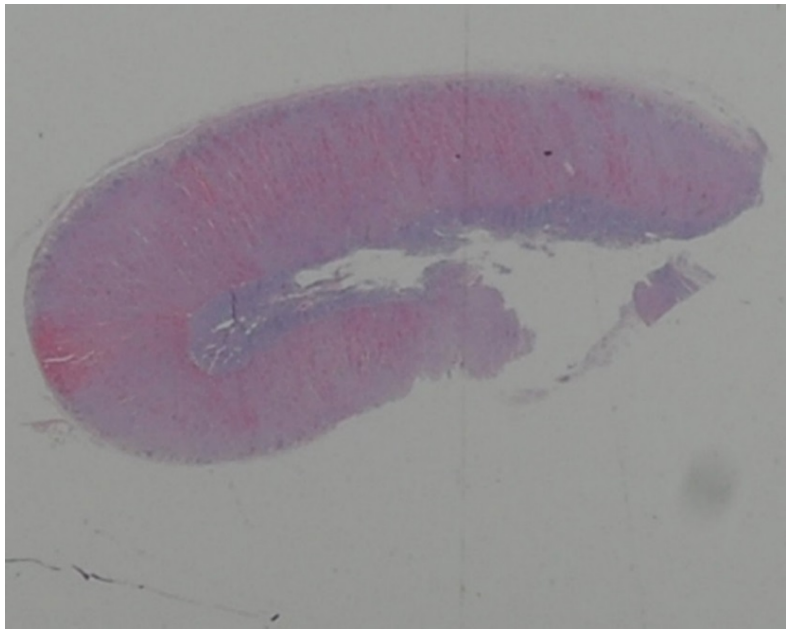

Figure 28a Gross histopathologic image of hemorrhage: not concentrated at corticomedullary junction, rather radiating out from medulla, all the way through the cortex, and concentrated in *zona glomerulosa*. Cortex:medulla ratio is good. Image courtesy of Dr. Kelly Joiner DVM, PhD, DACVP.

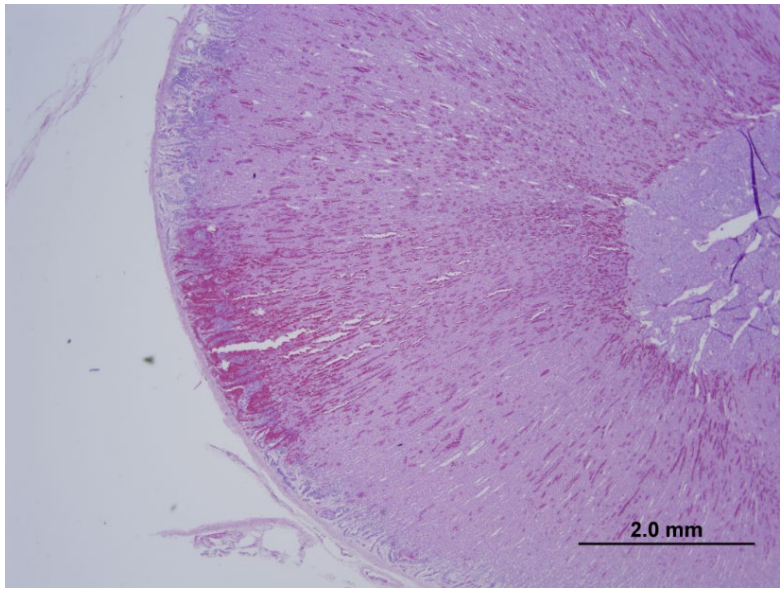

Figure 28b. Sub-gross histopathologic image of adrenocortical haemorrhage. Image courtesy of Dr. Kelly Joiner DVM, PhD, DACVP.

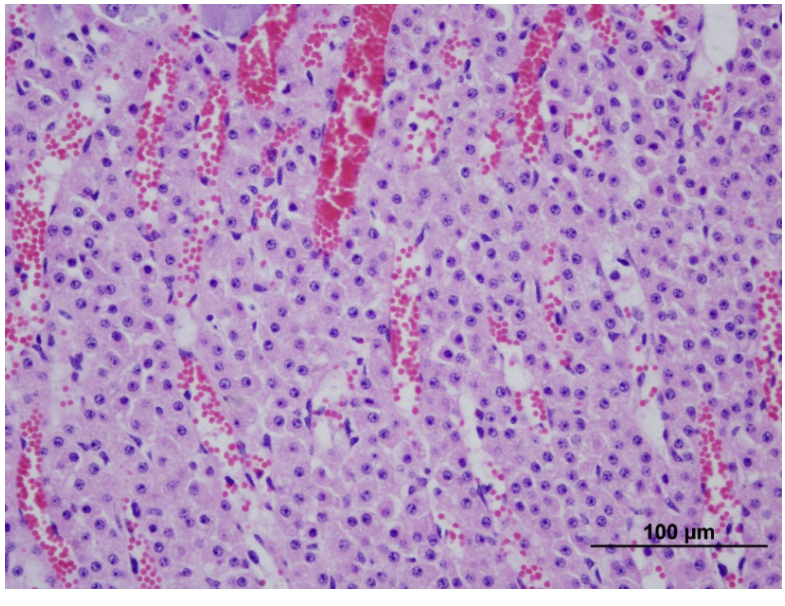

Figure 28c. Adrenocortical haemorrhage with some apoptotic cells. Image courtesy of Dr. Kelly Joiner DVM, PhD, DACVP.

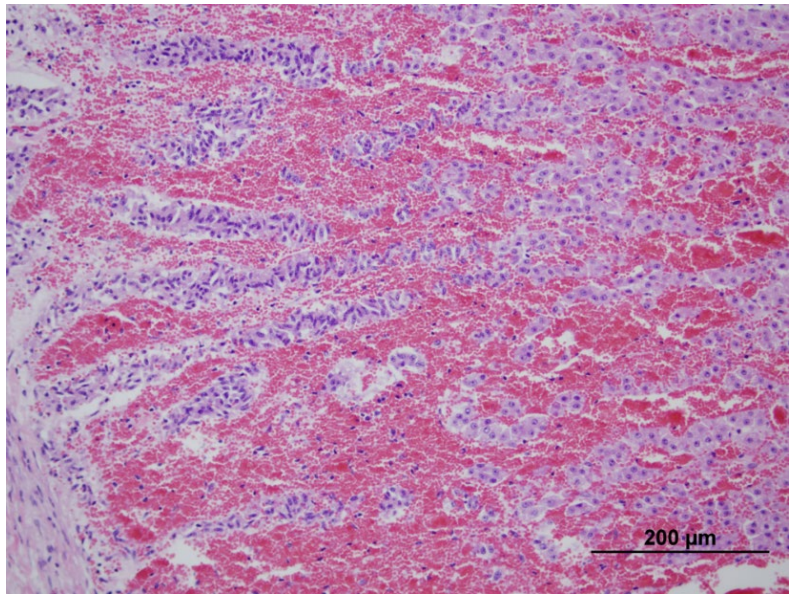

Figure 28d. Enlargement of *zona glomerulosa* haemorrhage. Image courtesy of Dr. Kelly Joiner DVM, PhD, DACVP.

4.8.3 Case number: 109612

A 15 year old Quarter Horse mare that presented for colic of one days duration. She had a heart rate of 80 beats per minute, purplish mucous membranes with a 2 second capillary refill time. She was estimated to be 10% dehydrated. A cecal impaction was identified by rectal palpation. The PCV was 64% and the total protein = 6.4 g/dL, with an albumin = 2.6 g/dL. The mare was leukopenic with a WBC count = 1,900 cells/µL, and neutropenic with 540 cells/µL. The SIRS score = 3. Abdominal fluid was a redish brown and bacteria were observed on cytology indicating a ruptured intestine. The horse was euthanized. Admission ACTH = 16.8 µg/dL and the ACTH/cortisol ratio = 10.1. A rupture of the base of the cecum secondary to a sever impaction was confirmed at necropsy.

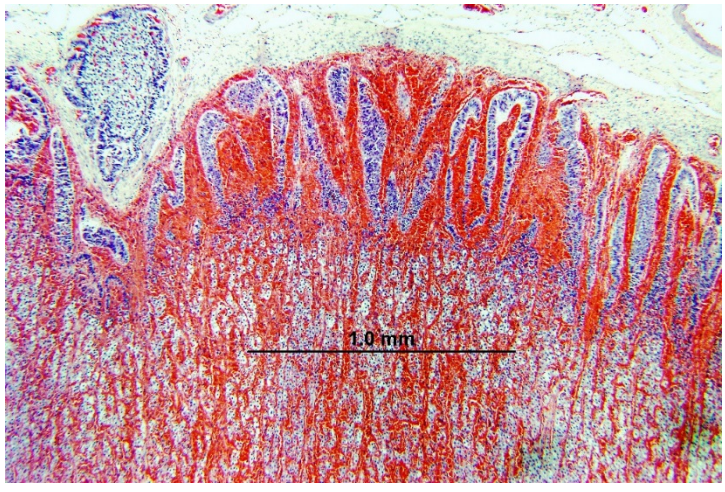

Figure 29a. There were moderate to marked acute multifocal to diffuse cortical haemorrhages in both adrenal glands. The cortices of both adrenal glands were thin and atrophied (the cortical to medullary ratio was approximately 1:6. Image courtesy of Dr. Richard C. Weiss VMD, PhD, DACVP.

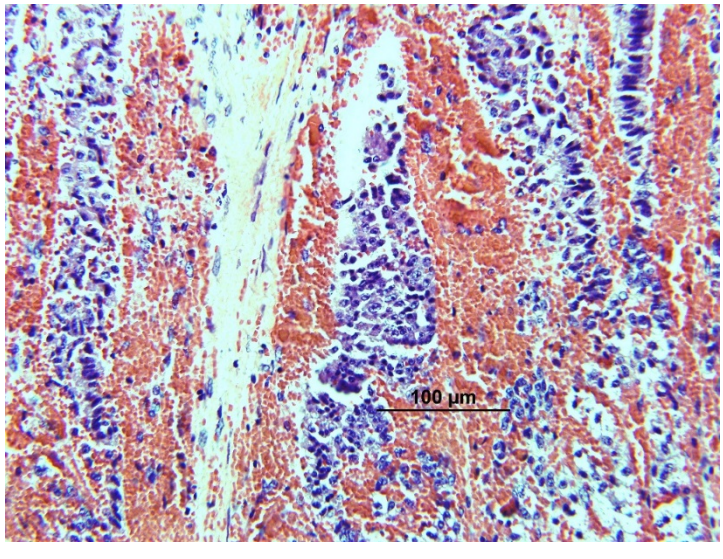

Figure 29b. Severe adrenocortical haemorrhage. Image courtesy of Richard C. Weiss VMD, PhD, DACVP.

5 General discussion

5.1 Validation of the ACTH stimulation test in healthy adult horses (I)

5.1.1 Maximal adrenal stimulation to low doses of cosyntropin

Healthy adult horses responded to extremely low doses of ACTH, administration of 0.02 µg/kg cosyntropin sufficient to significantly increase serum cortisol concentration 30 minutes after injection. As no significant differences were detected among maximum cortisol concentrations obtained after administration of cosyntropin at doses of 0.1, 0.25, and 0.5 µg/kg the lowest dosage to maximally stimulate the adrenal gland was 0.1 µg/kg. Higher dosages of cosyntropin resulted in a more prolonged adrenal response. The maximum adrenal response was obtained between 30 and 90 minutes after administration of 0.1 µg/kg cosyntropin, between 30 and 120 minutes after 0.25 µg/kg, and between 30 and 180 minutes after 0.5 µg/kg. The low dose of 0.1 µg/kg was selected to try and identify CIRCI in critically ill horses. In previous studies, the lowest dose of ACTH administered to adult horses was 0.5 5 µg/kg IV, and this dose resulted in cortisol concentrations higher than those obtained after maximum exertion during treadmill exercise (Marc *et al.*, 2000; Golland *et al.*, 1996).

When deciding to perform an ACTH stimulation test, the clinician is faced with a decision between using a cosyntropin dose high enough to obtain a significant increase in cortisol concentration, compared with baseline concentration, versus using a dose that will result in a maximum increase. Cortisol concentrations 30 minutes after of each of the cosyntropin doses used were significantly greater than baseline cortisol and concentrations obtained after administration of saline. However, although the 0.02 µg/kg dose resulted

in a significant increase in cortisol in all 8 horses, it did not cause maximum adrenal stimulation, in that peak concentration was lower than peak concentrations after administration of the other 3 higher doses. As no significant differences were detected in peak cortisol concentrations after administration of the 0.1, 0.25, and 0.5 µg/kg doses, we concluded that a dose of 0.1 µg/kg cosyntropin with analysis of cortisol concentration at T = 30 and 60 minutes can be used to assess maximum adrenal response in healthy horses. Use of a 0.1 µg/kg dose makes ACTH stimulation testing affordable for use in clinical cases, as one vial of cosyntropin (250 µg) is enough to perform five ACTH stimulation tests on 500 kg horses do long as the diluted cosyntropin is stored appropriately. Reconstituted cosyntropin can be stored in plastic for as long as 4 months in the refrigerator or as long as 6 months at –20ºC with no adverse effects on its bioactivity (Frank & Oliver, 1988).

For ACTH stimulation testing in dogs, there has been a shift from using a traditional dose of supraphysiologic dose of 250 µg/dog to using a dose of 5 µg/kg. The lowest dose of ACTH that causes maximum adrenal stimulation in dogs was found to be 0.5 µg /kg (Martin *et al.*, 2007). However the use of this lower dose, although more cost effective has not been readily adopted in canine practice. Evidence suggests that the lower dose of 0.5 µg /kg is at least as accurate and actually appears to be more sensitive at diagnosing CIRCI in critically ill dogs is emerging and is in agreement with studies in humans (Martin *et al.*, 2010; Soliman *et al.*, 2004; Beishuizen *et al.*, 2000). Although lower doses of cosyntropin will result in equal peak concentrations of cortisol as those obtained after high dose ACTH stimulation testing, the duration of increased cortisol concentrations is reduced when lower doses of cosyntropin are used in dogs as we found in horses and foals. Therefore the timing of blood collection of the post cosyntropin sample can be performed earlier, but must be more precise (Martin *et al.*, 2007; Kerl *et al.*, 1999).

5.1.2 No change in basal ACTH and cortisol concentrations between days

Mean baseline ACTH and cortisol concentrations did not differ between days of the study, ruling out day-to-day variations in environmental effects such as temperature, external stimulation, adrenal suppression from repeated ACTH administration or acclimatisation to the sampling protocol.

There was a statistically significant decrease in serum cortisol at T = 240 minutes after administration of the saline solution that was likely due to a reduction in endogenous stress levels. By that time, the horses were accustomed

to the procedure, and the environment was quieter at the time of blood sample collection.

5.1.3 Haematological changes in response to ACTH stimulation in adult horses

Statistically significant increases, compared with baseline, were found in the neutrophil/lymphocyte ratio at T = 120 minutes after the 0.02 µg/kg cosyntropin dose, at T = 240 minutes after the 0.1 µg/kg dose, and at T = 120 and 240 minutes after the 0.5 µg/kg dose. There were also increases in the neutrophil and total WBC counts 240 minutes after administration of both the 0.25 µg/kg and 0.5 µg/kg doses. In healthy horses circulating WBCs appear to be sensitive to subtle changes in the HPA axis. It is therefore advisable to collect blood for assessment of haematological data prior to performing an ACTH stimulation test. There was a doubling of the neutrophil/lymphocyte ratio between 0 and T = 240 minutes after the 0.25 µg/kg and 0.5 µg/kg cosyntropin doses. There was less variability with the 0.5 µg/kg dose, with an increase in the ratio by a factor of 1.5 and 2.4 times between 0 and T = 240 minutes. However, due to a lack of consistency between individual horses it is not advisable to use haematological parameters to assess adrenal responsiveness after cosyntropin administration. If more consistent, this would have provided a "stat-lab" assessment of adrenal function.

In a small previous study a relatively large dose of ACTH (approximately 2 µg/kg) was administered IV to 3 adult mares, and no significant differences were observed in total leukocyte or eosinophil counts at T = 30, 60, 120, and 240 minutes, compared with values for 4 control mares (Eiler *et al.*, 1979b). Differences were potentially not detected in the previous study because of the small sample size.

5.2 Validation of the ACTH stimulation test in healthy neonatal foals (II)

5.2.1 Maximal adrenal stimulation to low doses of cosyntropin

These results suggested that the lowest cosyntropin dose that can be used to assess adrenal gland function in healthy neonatal foals is 0.25 µg/kg, with peak cortisol response obtained between T = 20 and 30 minutes after cosyntropin administration. There were no significant differences between peak cortisol concentrations obtained in response to 0.25 and 0.5 µg/kg cosyntropin doses. In a study involving 3 to 4 day old foals, an equivalent increase in cortisol

concentrations occurred at T = 30 minutes after administration of 0.2, 2, and 5 µg/kg doses of cosyntropin. These authors suggested that a dose between 0.2 and 2 µg/kg be further evaluated to assess adrenal gland function in neonatal foals (Hart *et al.*, 2007). The present study investigated doses intermediate to these concentrations. In the study of 3 to 4 day old foals, the peak response to the 0.2 µg/kg dose occurred at T = 30 minutes, returning to baseline concentrations by T = 90 minutes (Hart *et al.*, 2007). In the present study, the peak response to the similar dose of 0.25 µg/kg was at T =20 minutes, with equivalent responses obtained between T = 10 and 60 minutes.

5.2.2 Rapid cortisol response to ACTH stimulation adrenal stimulation

Previous studies in adult horses (Stewart *et al.*, 2011) and 3 to 4 day old foals (Hart *et al.*, 2007) have not measured cortisol concentrations at times less than 30 minutes during an ACTH stimulation test. The study illustrates that the adrenal gland response to cosyntropin was very rapid and responses equivalent to the peak response occurred as early as T = 20 minutes after 0.25 and 0.5 µg/kg cosyntropin administration in the neonatal foals. This rapid response could shorten the time required to perform an ACTH stimulation test in healthy foals which would be advantageous in a busy clinical situation.

5.2.3 Lack of response to 0.02 µg/kg of cosyntropin

Administration of cosyntropin at a dose of 0.02 µg/kg was insufficient to increase serum cortisol concentrations in healthy neonatal foals. A similar result was found when eight 3 to 4 -day old foals received 1 µg of cosyntropin per /foal (approximately 0.02 µg/kg), and no significant increase in cortisol concentrations were detected (Hart *et al.*, 2007). Therefore we do not recommend using the 0.02 µg/kg dose for clinical testing of adult horses or neonatal foals.

5.2.4 HPA axis in neonatal foals compared to adult horses

It is essential that reference ranges for adult horses should not be used to assess adrenal gland function in foals. Neonatal foals lack a functionally mature and responsive HPA axis, and they have a lower cortisol response to exogenous or endogenous ACTH compared to adult horses (Hart *et al.*, 2009a; Rossdale & Ousey, 1984; Silver *et al.*, 1984). ACTH and cortisol concentrations are highest immediately after birth with a gradual decline during the initial week of life (Hart *et al.*, 2009a; Wong *et al.*, 2009b). Cortisol concentrations in the 2 to 12 day old foals in the present study were similar to those in 3 to 4 day old foals (Hart *et*

al., 2007). It is however very interesting that cortisol concentrations in foals are approximately one-third the values in adult horses from paper I (Stewart *et al.*, 2011). In comparison to the adult horses in paper I, the cortisol concentrations post ACTH administration were of lower magnitude, peaked earlier, and were of a shorter duration in foals (Stewart *et al.*, 2011).

The ACTH concentrations in adult horses from paper I and foals were similar (Stewart *et al.*, 2011). However the ACTH concentrations in our study were slightly less than reported in other studies of similarly aged healthy foals (Hart *et al.*, 2009a; Wong *et al.*, 2009b), however those studies used a chemiluminescent assay while the present study used an immunoradiometric assay, which may account for these slight differences (Schott, 2013).

5.2.5 Haematological changes in response to ACTH stimulation in foals compared to adult horses

In the foals of the present study, the neutrophil/lymphocyte ratio doubled between 0 and 120 minutes after administration of the 0.25 µg/kg cosyntropin dose, while in adult horses (paper I) the neutrophil/lymphocyte ratio increased significantly at T = 120 and 240 minutes after the 0.02, 0.1, and 0.5 µg/kg doses compared with baseline (Stewart *et al.*, 2011). The neutrophil and total WBC counts increased significantly at T = 240 minutes after both higher cosyntropin doses in adult horses but not in foals (Stewart *et al.*, 2011). Foals had significantly lower lymphocyte counts at T = 120 minutes after the 0.25 µg/kg dose, whereas lymphocyte counts did not change in adult horses (Stewart *et al.*, 2011). Circulating WBCs are sensitive to very subtle changes in the HPA axis in healthy foals and adult horses, with decreases in lymphocyte counts occurring in foals and increases in neutrophil counts documented in adult horses. The cause of this difference between the age groups is unknown. Similar to adult horses, it is advisable to collect blood for assessment of haematologic data in foals prior to administering cosyntropin. However, these haematological changes are transient and are not considered of any consequence to a healthy foal or a critically ill patient, as the ACTH stimulation test even at supra-physiological doses is considered safe enough to perform on the most severely ill children (Pizarro *et al.*, 2005). For a 50 kg foal receiving a dose of 0.25 µg/kg dose this is a total dose of 12.5 µg of cosyntropin per foal. Critically ill children, dogs and even cats have safely received a 250 µg of cosyntropin with no concern for side effects (DeClue *et al.*, 2011; Martin *et al.*, 2008; Pizarro *et al.*, 2005). It is likely that haematological changes would be more marked, prolonged and consistent in high-dose verses low-dose ACTH stimulation tests. This might even allow for the use of an indirect “in-clinic” assessment of adrenal function post cosyntropin

administration. If the neutrophil/lymphocyte ratio changed consistently after administration of a high dose of ACTH in normal animals then this could be used to assess adrenal function and bone marrow reserves in ill individuals using readily available hospital based benchtop haematological machines at any time, without having to measure hormone concentrations, which generally have to be performed by "office-hours only" laboratory personnel. Unfortunately, lack of consistency among individual foals precludes the use of CBC values to assess adrenal gland responsiveness after low dose cosyntropin administration. If changes were observed in haematological parameters after cosyntropin administration this would be useful, but the sensitivity of this as a diagnostic test appears to be low and some false negatives may occur.

5.3 Insulin dysregulation in horses with systemic inflammatory response syndrome (III)

5.3.1 Relative hypoinsulinemia occurs in horses with SIRS

Hyperinsulinemia was associated with a favorable outcome indicating a functional pancreas, where SIRS associated hyperglycaemia without hyperinsulinemia likely indicates pancreatic dysfunction and was associated with non-survival.

In patients with equine metabolic syndrome (EMS) hyperinsulinemia (either chronic or intermittent) is a component of insulin dysregulation and is usually associated with a poor prognosis as hyperinsulinemia has been shown to lead to laminitis (Bertin & de Laat, 2017; Menzies-Gow *et al.*, 2017; de Laat *et al.*, 2010; Frank *et al.*, 2010). Although hyperinsulinemia and obesity is well-described in horses, little was known about the function of the pancreas during illness in horses. Experimental LPS infusion resulted in increases in serum insulin, and this suggested that hyperinsulinemia occur in acutely sick horses (Tadros *et al.*, 2013; Toth *et al.*, 2008; Toribio *et al.*, 2005). In one study LPS infusion initially induced a mild decrease in insulin concentration suggesting inhibition of pancreatic β cell function before a more pronounced increase in insulin (Tadros *et al.*, 2013). In foals, as well as in other species severe systemic inflammation has resulted in relative hypoinsulinemia (Barsnick *et al.*, 2011; van Waardenburg *et al.*, 2006; Leininger *et al.*, 2000), where this was defined as a failure of insulin secretion in response to SIRS-induced hyperglycaemia. Transient *diabetes mellitus* was described in one foal with severe SIRS (Navas de Solis & Foreman, 2010). Our data was in agreement with those reports of relative hypoinsulinemia, in that severe hyperglycaemia, with a corresponding inadequate insulin response, was associated with non-survival. Although serum insulin was higher in survivors, it was rarely above reference range (20 μIU/mL) which suggests that rather than excessive insulin secretion in survivors, a

decreased insulin secretion was occurring in non-survivors. Therefore in our horses with SIRS, hyperglycaemia is likely caused by pancreatic dysfunction resulting in decreased insulin secretion and also peripheral tissue insulin resistance.

5.3.2 Hyperglycaemia and hypoinsulinemia were associated with non-survival indicating endocrine pancreatic dysfunction

Hyperglycaemia in horses with colic has previously been associated with poor outcome (Hassel *et al.*, 2009; Hollis *et al.*, 2007), and we found similar results. Large studies in human critical care units have shown that prolonged hyperglycaemia was associated with increased odds of infection, respiratory failure, cardiovascular dysfunction, and worse neurological status (Bochicchio *et al.*, 2005; Sung *et al.*, 2005; Capes *et al.*, 2000; Suematsu *et al.*, 2000). Causes of hyperglycaemia in critically ill patients include stress-associated cortisol secretion, hypoinsulinemia, increased gluconeogenesis and peripheral insulin resistance (Hassel *et al.*, 2009; Vanhorebeek *et al.*, 2007). In the present study, the serum insulin concentrations and glucose/insulin ratio suggest that horses with SIRS also have insulin dysregulation, at least partly as a consequence of peripheral tissue insulin resistance. In our study, the higher the SIRS score, and presumably the sicker the animal, the higher the serum glucose concentration, which suggests that the degree of hyperglycaemia could be used to estimate severity of SIRS in sick horses. However cautious interpretation is warranted, due to our small sample size and that no actual validation of the grouping system could be performed. Although, in study, non-surviving horses had a significantly larger SIRS score compared to surviving horses ($P < 0.05$). In human critical care serum glucose is used as a prognostic indicator, with associations made between specific outcomes and particular blood glucose threshold concentrations (Vanhorebeek *et al.*, 2007). However, a single parameter should never be relied upon to predict survival, and although serum glucose could be used to estimate disease severity, it should not be emphatically used to predict survival in sick horses based on its mediocre sensitivity and specificity in our sample of horses with SIRS.

The insulin/glucose ratio is used to estimate pancreatic β-cell secretion of insulin in response to a glycaemic challenge in horses (Bertin & de Laat, 2017). In the present study the insulin/glucose ratio was lower in non-survivors suggesting pancreatic dysfunction in non-survivors. Although no dynamic testing was performed to properly assess pancreatic function in our study, our data indicate that horses with SIRS undergo a transient dysfunction of the endocrine pancreas. The use of proxies, such as the insulin/glucose ratio, have not been fully validated in horses. However, this evidence of pancreatic dysfunction may provide an additional method of diagnostic assessment when attempting to identify multi-organ dysfunction (MODs) in critically ill horses. Pancreatic dysfunction has not previously been investigated when diagnosing MODs in critically ill horses (McConachie *et al.*, 2016). As the main stimulus

for insulin secretion in horses is high blood glucose, our data suggest that in non-survivors a reduction in insulin responsiveness and decreased insulin sensitivity may be responsible for the observed hyperglycaemia (de Laat *et al.*, 2016). A low serum insulin in non-survivors indicates a relative hypoinsulinemia and (potentially transient) endocrine pancreatic dysfunction. In contrast, although consistent with insulin dysregulation, the presence of mild hyperinsulinemia in the surviving horses demonstrates an appropriate pancreatic response. Endocrine pancreas dysfunction observed in our non-survivors could explain the lack of association between serum insulin and glucose those horses. The physiologically expected correlation between serum insulin and glucose was only observed in our surviving horses. This suggests that, only in survivors, SIRS associated hyperglycaemia leads to an appropriate endocrine pancreatic response resulting in rebound hyperinsulinemia, and thus limiting the potential deleterious effects of sustained hyperglycaemia. In the context of SIRS and critical illness, presence of mild hyperinsulinemia might be a good prognostic indicator as it implies an adequate endocrine pancreatic response to SIRS-associated hyperglycaemia.

5.3.3 Hyperinsulinemia and potential development of laminitis

In our study, no horse developed laminitis, therefore no conclusion could be made on the association between hyperinsulinemia and laminitis in the context of SIRS. As both hyperglycaemia and sepsis have been associated with laminitis; inflammation associated laminitis would be more likely a manifestation of MODs rather than a consequence of acute and mild hyperinsulinemia. Although similarities between inflammation-associated and insulin-associated laminitis have been documented at a molecular level, the different timings of the processes suggest that, if laminitis had been observed in our sample of sick horses, the severity of the disease process would have a greater effect on the induction of laminitis than the transient and mild hyperinsulinemia observed in our sick horses (Bertin *et al.*, 2013; de Laat *et al.*, 2011; Stewart *et al.*, 2009; Stewart *et al.*, 1995). In an experimental model, induction of hyperglycaemia was used to induce endogenous hyperinsulinemia (insulin = 208 ± 26.1 μIU/L), which was sufficient to create subclinical laminitis (de Laat *et al.*, 2012). As our surviving horses all had insulin concentrations < 55.2 μIU/mL and the non-survivors < 24.2 μIU/mL, marked hyperinsulinemia sufficient to lead to laminitis did not occur in our patients. Whereas inflammation is one of the underlying causes of laminitis in sick horses, we had questioned if hyperinsulinemia in sick horses would be one of the underlying causes of laminitis in this population. It seems unlikely from our results, but as there were no cases of inflammatory laminitis in which insulin was measured this question remains unanswered.

5.3.4 Limitations of the study

Assessment of endocrine pancreatic dysfunction was limited to measurement of glucose and insulin concentrations and no dynamic testing was performed during the episode of SIRS. As EMS is based on a typical phenotypic presentation (in addition to identification of insulin dysregulation) and the majority of horses with clinical PPID have hirsutism, there were a few clinical cases of PPID and EMS that were excluded from this study. As dynamic tests such as the insulin response test, oral glucose test, and ACTH response to thyrotropin-releasing hormone stimulation would have been required prior to, or after the period of illness to completely rule out subclinical EMS or PPID (de Laat & Sillence, 2016; Bertin & Sojka-Kritchevsky, 2013; Beech et al., 2011) This study involved spontaneous disease in client-owned animals, where survivors were discharged from hospital after recovery, it was therefore impossible to perform dynamic testing prior to, or (in most cases) after the onset of illness for the purpose of this study. There was no evidence of pituitary enlargement in the non-surviving horses in which a necropsy was performed, suggesting that only a very limited number of horses with PPID could have possibly been included in our sample population. Further work to validate the effects of dynamic tests of endocrine pancreatic function in sick horses could potentially be explored in future studies.

5.3.5 Insulin infusions to control SIRS induced hyperglycaemia and potential risk of laminitis

As recommended in guidelines for human medicine (Jacobi et al., 2012), cautious insulin therapy might be of benefit for horses in SIRS presenting with severe unregulated hyperglycaemia. Tight glycaemic control through insulin infusions and frequent monitoring, with maintenance of serum glucose concentrations between 80 – 110 mg/dL (4.4 – 6.1 mmol/L is no longer recommended in critically ill humans due to the side effects of inadvertent hypoglycaemia (Macrae *et al.*, 2014). Less stringent conventional glucose regulation, such as between 140 – 180 mg/dL (7.8 – 10 mmol/L) through insulin infusions resulted in an improvement of outcome with a decreased incidence of hypoglycaemia in critically ill humans (Griesdale *et al.*, 2009; Preiser *et al.*, 2009; Arabi *et al.*, 2008).

Our laboratory reference range for blood glucose in horses is 80 – 124 mg/dL (4.4 - 6.9 mmol/L), and a continuous rate infusion of insulin could be administered to hyperglycaemic critically ill horses in an attempt to control blood glucose to within reference range. We routinely do this for hyperlipaemic patients with simultaneous infusions of glucose and insulin. Both groups of patients are considered insulin resistant, therefore reasonably high dosages of

insulin are required to control glucose concentrations than needed in normal patients, however the dosages of insulin administered are less than that administered experimentally to induce laminitis (de Laat *et al.*, 2010) In dogs with diabetic ketoacidosis a dose of insulin of 0.1 IU/kg/hr (by adding 5 IU/kg of insulin to a 500 ml bag of 0.9% NaCl (isotonic saline) and administered at 10 mL/hr is recommend to reduce hyperglycaemia (Dethioux & Goy-Thollot, 2010). Adding 2 mL of plasma per 50 mL of saline solution prior to adding the insulin prevents adhesion of insulin to the synthetic plastic material of the tubing and bag (Dethioux & Goy-Thollot, 2010) I have used this dosage in hyperlipaemic ponies and critically ill neonatal foals that are receiving intravenous dextrose or partial or total parenteral nutrition to control hyperglycaemia by forcing glucose uptake by peripheral tissue. Glucose concentrations are measured every 4 hours and if the animal becomes hypoglycaemic the rate of the insulin infusion is decreased, while if the animal becomes hyperglycaemic the rate of glucose infusion is decreased. This dose is approximately one quarter of that used to induce laminitis experimentally in non-insulin resistant (normal) horses and ponies (personal observation). Laminitis has been experimentally induced by administration of a bolus of 45 mIU/kg of insulin followed by a constant rate infusion of 6 mIU/kg/minute (0.36 IU/kg/hour) with a glucose infusion administered at a variable rate to ensure euglycaemia (euglycaemic-hyperinsulinemic clamp technique). This resulted in massive serum insulin concentrations > 1000 µIU/mL (de Laat *et al.*, 2010).

In another experimental study in which mild hyperglycaemia (10 ± 0.78 mmol/L) was induced by administration of 50% dextrose over 48 hours at a rate of 0.68 mL/kg/hr (with reductions in the infusion rate of 10% if serum glucose exceeded 15 mmol/L), endogenous hyperinsulinemia (insulin = 208 ± 26.1 µIU/L) was created which induced lamellar lesions but not foot pain (de Laat *et al.*, 2012). Therefore although experimental super-physiologic infusions of insulin can induce laminitis, prolonged experimental hyperglycaemia also induces sub-clinical lamellar changes. Thus leaving equine patients in a hyperglycaemic state is also potentially a risk factor for laminitis. Therefore, in addition to commencing with the protocol used for ketoacidotic diabetic dogs, daily measurement of serum insulin concentrations in equine patients to ensure that they remain well below 50 µIU/L during the infusions would be advisable. More research documenting the use of insulin infusions in non-terminal hyperglycaemic equine critical care cases with serial monitoring of serum insulin concentrations is warranted (Figure 30). It would also be interesting to prospectively examine insulin and glucose concentrations in critically ill horses that develop laminitis; however with supportive care including digital hypothermia and use of anti-inflammatory drugs the incidence of laminitis appears to have reduced (personal observations). Over the last 20 years, having worked in various regions with differing equine population and diseases, the

variation in incidence in laminitis observed by this author is likely a reflection of the multifactorial aspects of genesis of laminitis.

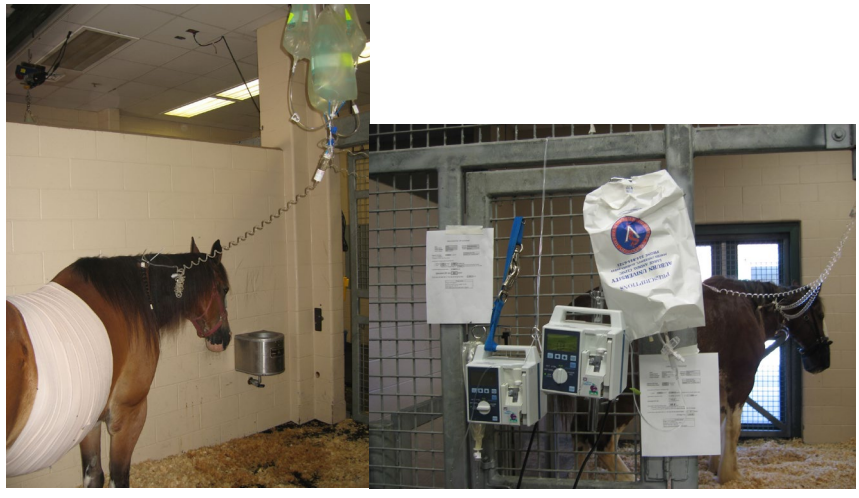

Figure 30. A post-operative patient in the equine intensive care unit receiving high volume intravenous fluid therapy in conjunction with a glucose and insulin infusion. Photos: A.J. Stewart.

5.4 Cortisol and ACTH concentrations in horses with systemic inflammatory response syndrome (IV)

5.4.1 The stress response in horses with SIRS

ACTH and cortisol concentrations were significantly higher in non-surviving horses and those with an ischemic gastrointestinal lesion. This was not unexpected, as the presence of an ischemic gastrointestinal lesion and non-survival are indicative of more severe disease processes, and this is compatible with a physiologic response to stress. The ACTH/cortisol ratio was significantly higher in non-surviving horses, horses with SIRS and those with ischemic lesions. The high ACTH/cortisol ratio indicated that the magnitude of the pituitary response was greater than that of the adrenal response, and may suggest possible RAI/CIRCI (Gold *et al.*, 2007).

Equine emergency patients experience stress from fear, pain, dehydration, inappetence and the consequences of systemic inflammation such as fever, hypotension or hypoxemia. Factors shown to affect CRH secretion in a variety of species include cytokines, lipopolysaccharide, (brain-derived neurotropic factor, neurosteroids, glutamate, γ-aminobutyric acid, nitric oxide and through

the negative feedback effect of glucocorticoids (Dembek & Toribio). Lipopolysaccharide was detected in 29% of horses presenting for colic, and is more commonly detected in horses with ischemic gastrointestinal lesions (Senior *et al.*, 2011). Interleukins-1β, -6 and -8 mRNA expression have been shown to be upregulated in surgical colic patients (Epstein *et al.*, 2016). Although neurosteroids have been measured in critically ill foals, the direct effect of various other inflammatory mediators on the HPA axis of sick horses has not been investigated. Arginine vasopressin and CRH stimulate release of ACTH from corticotropes in the pituitary *pars distalis*, and this is moderated by cytokines, lipopolysaccharide, nitric oxide and glucocorticoids during illness (Dembek & Toribio). Although we did not assess CRH or vasopressin concentrations in this study, the prevalence of ACTH hyposecretion or hypothalamic dysregulation in relation to HPA axis dysfunction is low compared to adrenocortical failure in humans and septic foals (Annane *et al.*, 2018; Hurcombe *et al.*, 2008).

5.4.1 Association between ACTH and survival

Studies assessing ACTH concentrations in sick adult horses are sparse, with mean ACTH concentrations being 2.4 times higher in 43 horses with colic compared to normal controls, with the highest values occurring in horses with strangulation of the small or large intestine (Ayala *et al.*, 2012). Similarly, we found that ACTH was significantly higher in horses with ischemic gastrointestinal lesions than those without. The median ACTH concentration in non-surviving horses was 3.6 times higher than in normal horses (from our institution measured with the same assay (Funk *et al.*, 2011)). However, individual variation in the sick horses was extreme, with the highest ACTH value occurring in a surviving horse, which was 49 times higher than normal horses. When ACTH concentrations were within reference range at either admission or on day 2, this was associated with a higher odds ratio of survival.

5.4.2 Clinical implications for testing for PPID in horses that have recovered from illness

Although the pituitary gland has a high capacity to increase ACTH production, in surviving animals this response was rapidly normalized, as over 90% of horses had an ACTH concentration within reference interval prior to patient discharge. This has clinical implications for the diagnosis of PPID. If PPID is suspected in an older hospitalized horse testing for PPID may be recommended prior to patient discharge. In the present study, ACTH concentration was normal in 95/103 (92%) of horses at the time of patient discharge, with 5 horses having an ACTH between 28-40 pg/mL and only 3 horses having an ACTH concentration between 60-110 pg/mL. Therefore at the time of patient discharge ACTH testing for PPID could be reliably performed in 92% of cases. Therefore if there was concern about a differential of PPID in a horse that had recovered from illness,

then collection of plasma to measure ACTH concentration for the purpose of diagnosing PPID, could be performed prior to discharge, or at least the horse could be hospitalized for a further day prior to sample collection. There would only be an 8% chance of a false positive result it testing was performed on the day of discharge.

There is reemerging concern about the stability of equine ACTH (Bertin *et al.*, 2018b). Our current recommendation is to chill samples immediately, remove plasma by centrifugation within 2 hours and analyse a non-previously frozen sample within 8 hours of collection, because of the high proportion of misdiagnoses that can occur due to unpredictable deceases and increases in measured ACTH concentrations with less than ideal sample handling procedures which can result in false positive and negative diagnoses of PPID (Bertin *et al.*, 2018b). Therefore measurement of ACTH concentrations in some situations will be best performed at institutions with onsite laboratories rather than shipping samples overnight from remote private practices or batch freezing samples prior to analysis. Based on the results from the current study (paper IV), there would only be an 8% chance of a false positive result for the diagnosis of PPID if testing was performed on the day of patient discharge after recovery from illness, which is actually lower than the number of misclassifications that were identified in the diagnosis of PPID when samples were mishandled (Bertin *et al.*, 2018b).

5.4.3 Comparison of ACTH concentrations in sick adult horses to foals

In comparison to foals, adult horses tend to have lower ACTH concentrations, but ACTH is higher in septic compared to healthy foals and higher in non-survivors than survivors (Stewart *et al.*, 2013; Hurcombe *et al.*, 2008; Gold *et al.*, 2007). As measured ACTH concentrations vary when using different analytical methodologies, it is difficult to directly compare actual values obtained in different studies. The Scantibodies™ immunoradiometric kit used in the present study has been shown to be highly accurate, but measures 3.6-fold lower than the chemiluminescent Immulite™ (Schott, 2013).

5.4.4 Association between cortisol and survival

The *zona fasciculata* in the adrenal cortex secretes glucocorticoids (cortisol), which decrease CRH, vasopressin and ACTH secretion by negative feedback. Cortisol increases gluconeogenesis, decreases glucose utilization, increases serum glucose and inhibits the effects of insulin. Cortisol concentrations were increased for each of our three outcome variables (non-survival, presence of SIRS or ischemic gastrointestinal lesion). These findings were supported by previous studies in horses with colic (Ayala *et al.*, 2012; Hinchcliff *et al.*, 2005). Mair showed that horses with colic are more likely to have a serum cortisol > 7.25 µg/dL than healthy controls, that cortisol is higher in colic patients with a heart rate > 45 beats per minute, and that horses with a higher cortisol demonstrate more severe signs of colic, although a direct association

between high cortisol and survival was not detected due to wide individual variations in cortisol concentration in both survivors (2.8 to 47.6 µg/dL) and non-survivors (5.1- 38.8 µg/dL) (Mair *et al.*, 2014).

5.4.1 Comparison of cortisol concentrations in sick adult horses to foals

Our previous work comparing normal 2-12 day old foals and adult horses showed that baseline cortisol values are lower in normal neonatal foals than adult horses, but adult horses appear to have a greater adrenal capacity and reserve than neonatal foals to produce and release cortisol after exogenous ACTH stimulation (Stewart *et al.*, 2013; Stewart *et al.*, 2011). This also appears to be true in illness, with our sick adult horses generally having higher cortisol concentrations than those observed in septic neonatal foals (Hurcombe *et al.*, 2008; Gold *et al.*, 2007).

5.4.2 Limitations of immunoassays compared to liquid–chromatography mass spectrometry

Our previous work comparing normal 2-12 day old foals and adult horses showed that baseline cortisol values In humans with septic shock, routinely used immunoassays for total cortisol appear to correlate poorly (inter-assay variations in up to 27% of cases), when compared to the gold standard of liquid–chromatography mass spectrometry (LC-MS). Unfortunately LC-MS is time and labor intensive and not available for clinical cases at most institutions (Bornstein, 2009; Briegel *et al.*, 2009) Although there are limitations in the use of immunoassays to measure cortisol and ACTH concentrations, standardized sample handling procedures for samples and looking at trends and relative changes in hormone concentrations measured on same assay from the same institution are likely to be valid.

5.4.3 Scoring systems for severity of illness and clinical implications equine for colic patients

There is no accepted grading scheme to categorize the severity of illness in sick adult horses, although a scoring system for multiple organ dysfunction in equine surgical patients has recently been developed (McConachie *et al.*, 2016). Sepsis is a common cause of hospitalisation in neonatal foals, and several scoring systems have been compared to categorize the severity of illness in septic foals or assess the likelihood of survival, which allows reasonable comparisons of illness severity between various research studies (Wong *et al.*, 2018; Dembek *et al.*, 2014). We have utilized the number of SIRS criteria to try and objectively categorize the severity of illness in sick adult horses (Bertin *et al.*, 2018a) Although the median SIRS score was higher in non-survivors than survivors, there was only a trend for the SIRS score to be

higher in horses with ischemic lesions compared to those with non-ischemic lesions. SIRS score may not be the best method to classify the severity of illness in equine colic cases. The presence of an ischemic lesion in a colic case will be fatal unless surgically removed, but the severity of illness at patient admission is reflected by the location, extent and duration of the area of devitalised bowel and by any supportive treatments such as fluid therapy, analgesics, anti-inflammatories and endotoxin binders.

5.4.4 Clinical implications of using cortisol and ACTH as a predictors of survival

In severely ill humans, the APACHE II score has been used within 24 hours of patient ICU admission to assess illness severity and risk of death (Knaus *et al.*, 1985) However the day 2 cortisol concentration was a better discriminator of ICU patient outcome with a predictive ability of 81%, while the APACHE II score had a predictive ability of only 70% (Rothwell & Lawler, 1995; Jarek *et al.*, 1993). The use of cortisol, ACTH and ACTH/cortisol ratios as predictors of survival has not been previously assessed in a large group of horses admitted to an equine emergency service. Although no single factor should ever be used to predict survival of individual cases, and experienced clinicians have been shown to be better predictors of survival than soring systems, (Rohrbach *et al.*, 2006) the relative importance of these factors and potential clinical implications should be considered. Although many studies have investigated admission clinicopathologic findings to assess likelihood of survival, in many cases changes over time in patients surviving the initial admission period (or those not euthanized due to a poor prognosis, inhumanity or lack of financial resources to justify the high costs involved in critical care of the equine patient) are more important. We attempted to assess cortisol and ACTH concentrations over 6 days of hospitalisation, but many non-severely ill horses were discharged prior to day 6, whereas the majority of our non-survivors were euthanized within the initial 24-hours, with only 11 non-survivors available for testing on day 2. Thus the study lacked sufficient power to detect if there was a difference in cortisol or ACTH concentrations between survivors and non-survivors on days 2, 4 and 6 of hospitilisation. At patient admission many factors beyond the effects of illness may affect ACTH and cortisol concentrations such as fear from transportation, a foreign indoor environment, rapidly moving support personnel and venipuncture in addition to effects of sedation and analgesics. Blood samples were collected prior to hospital administration of any medication but there was no attempt to make allowances for distance travelled or pre-admission medication, although none of the horses appeared to be sedated. Mair previously found that the effect of transport was not a significant confounding factor influencing cortisol concentrations in horses with colic (Mair *et al.*, 2014) When investigating effects of spontaneous disease in admitted patients these factors cannot be controlled, but are representative of a typical clinical scenario.

5.4.5 Inter-relationships between the HPA axis and insulin/glucose dynamics

In severe illness, SIRS, sepsis, shock and multiple organ dysfunction occurs resulting in loss of physiologic hemostatic mechanisms. Cortisol should inhibit insulin in order to raise serum glucose concentrations, but hyperglycaemia has shown to be a poor prognostic indicator in horses with colic (Hassel *et al.*, 2009). We have recently shown that hyperinsulinemia and hyperglycaemia are both common in equine patients diagnosed with SIRS, but that horses with relative hypoinsulinemia and corresponding hyperglycaemia have a worse prognosis, due to suspected endocrine pancreatic insufficiency (Bertin *et al.*, 2018a).

5.4.6 High ACTH/cortisol ratio as an indicator of adrenal dysfunction

Pituitary reserve and ability to release large amounts of ACTH with the stress of illness appears to be greater in horses than the corresponding capacity for adrenal production and release of cortisol, resulting in a high ACTH/cortisol ratio, and has been defined as ACTH-cortisol imbalance (Dembek *et al.*, 2017). In cases when the cortisol release is inadequate for the degree of illness because of adrenal gland dysfunction, then a diagnosis of RAI/CIRCI can be made. Although the ACTH/cortisol ratio is not as accurate as results from an ACTH stimulation test, it is easy to perform, less expensive and has provided useful information in critically ill foals (Hart *et al.*, 2009b; Wong *et al.*, 2009a; Gold *et al.*, 2007). Our results showed that the ACTH/cortisol ratio was higher in non-survivors, horses with SIRS and those with ischemic lesions. This was similar to results found in some studies involving septic foals where the ACTH/cortisol ratios were significantly higher in septic foals than sick-non-septic or normal foals (Dembek *et al.*, 2017; Gold *et al.*, 2007). The actual value of the ratio was much higher in foals compared to adult horses because healthy and sick foals tend to have a higher ACTH and lower cortisol concentrations than adult horses (Stewart *et al.*, 2013; Stewart *et al.*, 2011) An appropriate cortisol concentration in response to illness of varying severities has not previously been defined for adult horses. In humans and foals a low baseline cortisol concentration or an inadequate response to a high or low dose-ACTH stimulation test have been advocated as means to diagnose CIRCI (Annane *et al.*, 2018; Hart *et al.*, 2009b; Wong *et al.*, 2009a)

As seen in this study, the most common reason for emergency hospital admission for horses is colic, with ischemic bowel requiring prompt surgical intervention under general anesthesia. Inflammation, shock, coagulation disorders, ischemia/reperfusion injury, SIRS and ileus are common syndromes requiring multimodal medical therapy in colic patients. Although ACTH and cortisol are significantly higher in horses with ischemic gastrointestinal lesions and non-surviving horses, the ACTH/cortisol ratio was significantly higher in non-surviving horses, horses with SIRS and those with ischemic lesions. The

high ACTH/cortisol ratio may indicate pituitary-adrenal dysregulation or an inadequate cortisol response to the stress of illness suggesting possible RAI/CIRCI(Gold *et al.*, 2007) Further investigation is required to determine if low-dose glucocorticoid therapy is beneficial in improving survival in a subset of adult equine intensive care patients.

5.5 Identification of adrenal dysfunction in critically ill adult horses

5.5.1 Summary of findings

Similar to our findings in project (IV) which included a much larger group of horses, the basal concentrations of ACTH and cortisol were statistically higher at admission in non-survivors than survivors in this group of equine hospital emergency admissions. Although horses with ischemic gastrointestinal lesions had statistically higher basal cortisol concentrations at admission than those without ischemic lesions, those with ischemic lesions failed to respond to a low-dose ACTH stimulation test. In contrast, horses without an ischemic lesion did respond, with higher cortisol concentrations at T = 30 minutes after cosyntropin administration compared to basal cortisol. Similarly, there was a tendency for non-surviving horses to fail to respond to the low-dose ACTH stimulation test compared to surviving horses. Adrenal insufficiency was diagnosed in 12 critically ill horses fulfilling our criteria for RAI/CIRCI.

5.5.2 A diagnosis of CIRCI must only be made in critically ill horses

Many more horses had a low basal or delta-cortisol concentrations at various times throughout hospitalisation and this was considered adrenal dysregulation. Although all these cases were sick, they were not all considered to be critically ill, and hence not classified as CIRCI cases. CIRCI only occurs by definition, in critically ill patients (Annane *et al.*, 2018). However, many of our cases certainly did have cortisol or delta cortisol concentrations less than not only what was expected for their degree of illness, but in many cases, less than that of normal quiet teaching horses (Stewart *et al.*, 2011). Therefore this is considered to be due to some degree of adrenal dysregulation. However, a numerically low basal-cortisol or poor response to ACTH stimulation should not be considered on its own as a defining criteria for a diagnosis of CIRCI in all horses admitted to a referral emergency service.

Adrenal insufficiency is considered common in human patients with sepsis, septic shock, severe trauma and acute respiratory distress syndrome, however the incidence is dependent on the population studied and the diagnostic test and criteria used to make the diagnosis (Annane *et al.*, 2018). Although all equine cases included in this study were presented to referral hospitals as emergency

admissions, the severity of illness varied greatly, and the numerical cut-offs used for an inappropriately low cortisol may not have been appropriate for horses with relatively mild illness such as impactions of the small and large colon, or large colon displacements that did not have any ischemic lesions. However, why their cortisol concentrations were lower than normal non-stressed horses requires further investigation.

5.5.3 Defining a cut-off value for an inappropriately low basal cortisol concentration in critically ill patients

Mean basal cortisol in surviving human ICU patients was 27 µg/dL, compared to 47 µg/dL in non-survivors, with cortisol found to be an independent predictor of outcome (Rothwell & Lawler, 1995). Others have found mean cortisol in human patients with septic shock to average 50 µg/dL (Marik & Zaloga, 2002). Although there has been no direct comparison of the adrenal capacity of horses compared to humans, the values we obtained in surviving and non-surviving ICU horses were lower. The maximum cortisol concentration was 55.9 µg/dL in a non-surviving horse. However all our patients were ambulatory and unlikely to be as sick as human or neonatal foal ICU patients with septic shock. Compared to neonatal foals, septicemia is uncommon in adult horses, and although traumatic septic arthritis is common, these horses are rarely systemically ill and were not included in the sick horse population in this study. Our population included mostly colic cases with ischemic and non-ischemic gastrointestinal lesions, with a lower number of critically ill horses with colitis and pleuropneumonia. Therefore, we were interested in the outcome variables of ischemia, SIRS and survival rather than sepsis or septic shock as has previously bene described in neonatal foals (Hart *et al.*, 2009b; Wong *et al.*, 2009a).

There is still a lack of consensus, even among a panel of experts, as to the best methods to diagnose CIRCI in humans (Annane *et al.*, 2018). Marik suggested that random cortisol concentrations < 25 µg/dL in highly stressed patients was useful to diagnose adrenal insufficiency and substantiate hydrocortisone therapy (Marik & Zaloga, 2003). The European CIRCI guidelines suggest using either a random endogenous cortisol of < 10 µg/dL or a delta-cortisol of < 9 µg/dL after intravenous administration of 250 µg cosyntropin (high-dose ACTH stimulation test) (Annane *et al.*, 2018). Patients diagnosed as having CIRCI using these criteria had poorer outcomes than those without CIRCI (Annane *et al.*, 2018).

5.5.4 Defining a cut-off value for an inappropriately low basal cortisol concentration in critically ill adult horses

In healthy unstressed adult horses we previously found a basal cortisol of 6.15 ± 1.63 µg/dL (Stewart *et al.*, 2011). However, sick animals should have higher

cortisol concentrations than healthy animals. To simulate a physiologically stressed basal cortisol, Hart used the lowest (mean – 1 SD) cortisol concentration obtained after administration of cosyntropin to healthy foals to define a cut-off value for an appropriate basal cortisol concentration in sick foals (Hart *et al.*, 2009b). Using this definition, Hart found that 46% of hospitalized foals had inappropriately low basal cortisol (however not all of these foals were septic or critically ill). Using the same definition, using data from normal adult horses, this results in a cut off value in sick adult horses for an inappropriately low basal cortisol concentration of < 9.7 µg/dL. In our study, the number of hospitalized adult horses with basal cortisol < 9.7 µg/dL, was 55% at admission, 79% on day 2, 94% on day 4 and 100% on day 6. However, this is likely too high a cut-off value and not useful as diagnostic criteria for diagnosing RAI/CIRCI in sick hospitalized adult horses. Therefore I elected to use a far more conservative approach, by using a basal cortisol concentration less than the lowest (mean -1SD) obtained from normal horses to define an inadequate basal cortisol in sick horses with a cut-off value of < 4.51 µg/dL, which was identified in 45% of horses; 7% at admission, 10% on day 2, 29% on day 4 and 64% on day 6. The high number of sick horses with basal cortisol concentrations less than the lowest basal cortisol concentration detected in healthy (quiet temperament university owned) horses was a surprising finding. Also, that the proportions of horses with an inappropriately low basal cortisol concentration increased throughout hospitalisation was even more interesting. By day 6 the sickest horses were dead and the healthiest horses had been discharged. The remaining horses were relatively compromised cases, and therefore possible explanations include adrenal structural damage, utilization of cholesterol substrate to synthesise cortisol, adrenal exhaustion, or negative feedback from previously elevated cortisol concentrations.

5.5.5 Defining a cut-off value for an inappropriately delta cortisol concentration in critically ill patients

Traditionally, the dynamic high-dose ACTH stimulation test is used to diagnose absolute adrenal insufficiency (Addisons's disease), in which supraphysiological doses of ACTH are administered, e.g. one entire vial of synthetic ACTH which is 250 µg /human patient or approximately 2-5 µg/kg. This dose creates ACTH concentrations 100-fold greater than secreted endogenously in response to stress *(Oelkers, 1996).* Critically ill patients are considered to have relative rather than absolute adrenal insufficiency, and a normal response to a high-dose ACTH stimulation test does not rule out RAI/CIRCI, as RAI is believed to be partly due to adrenal resistance to ACTH. A supraphysiologic dose of ACTH can override adrenal resistance to ACTH, resulting in a normal cortisol response in some cases *(Oelkers, 1996).* Although the high-dose ACTH stimulation test has remained the most popular, the physiologic low-dose (1-2 µg/human) ACTH stimulation test was introduced

to increase the test sensitivity. It appears more sensitive at diagnosing early/mild cases of RAI/CIRCI, (Soliman *et al.*, 2004) and accordingly, has been recommended by some as the preferred test (Gonc *et al.*, 2003; Beishuizen *et al.*, 2000). One randomized trial showed that the low-dose (1 µg) ACTH test was better able to predict the longer duration of vasopressor dependence and the hemodynamic response to hydrocortisone in septic shock patients than the high-dose ACTH test or a random basal cortisol < 25 µg/dL, in extremely ill people with a mortality rate of 47% (Marik & Zaloga, 2003). A prospective study with 74 septic shock patients showed that the high and low-dose ACTH stimulation tests were equally accurate in predicting dependency on vasopressors and mortality (Moraes *et al.*, 2012).

The latest European CIRCI guidelines suggests to utilize high-dose rather than low-dose ACTH stimulation to diagnose CIRCI, but the main reason was due to convenience (Annane *et al.*, 2018). The high-dose test is safe and easy to perform, as an entire vial of cosyntropin is used. The European task-force stated that the major disadvantage of the low-dose test was that it required additional preparation to dilute the ACTH. (This was a surprising recommendation as syringing reconstituted cosyntropin using the same 10 ml syringe used in the reconstitution process into a 250 mL bag of 0.9% NaCl and doing a basic mathematical calculation is really not that difficult!) Equal efficacy (or increased sensitivity as shown in some studies); decreased physiological effects of the test itself (haematological changes); and lower cost all provide reasons to perform the low-dose ACTH stimulation test in preference to the high-dose test. In human medicine there is a huge amount of medical waste and inefficiency. Diluted cosyntropin in a now unsealed bag of 0.9% NaCl would be unlikely be allowed to be saved for future testing on additional patients, even if sterilely prepared and appropriately stored.

5.5.6 Defining a cut-off value for an inappropriately delta cortisol concentration in sick adult horses

As the 0.1 µg/kg dose of cosyntropin was shown to result in maximal adrenal stimulation in healthy horses compared to 0.25 and 0.5 µg/kg, we elected to utilize this low-dose, with post cosyntropin cortisol sample collected after 30 minutes to correspond with the peak cortisol response. The mean ± 1SD delta-cortisol was 5.65 ± 1.50 µg/dL 30 minutes after 0.1 µg/kg (Stewart *et al.*, 2011). Higher doses of cosyntropin result in a more sustained adrenal stimulation, which would allow post cosyntropin samples to be collected over a wider testing window (Stewart *et al.*, 2011). However, at a dose of 0.1 µg/kg, one 250 µg vial of cosyntropin is sufficient to perform five ACTH stimulation tests on 500 kg horses, making the test affordable even for large equine patients. Due to the large range of body weights of horses, we elected to also use a µg/kg dose rather than a µg/horse dose.

Utilizing a variation of Hart's formula, we defined an inappropriate delta-cortisol response to a low-dose ACTH stimulation test as a value less than the

mean delta-cortisol obtained from healthy horses 30 minutes after administration of 0.1 µg/kg of cosyntropin, which was a delta-cortisol < 5.65 µg/dL (Stewart *et al.*, 2011; Hart *et al.*, 2009b). We also described a more conservative cut-off value of less than the mean -1SD delta-cortisol obtained from healthy horses, with a value of < 4.15 µg/dL. Hart found that 52% of hospitalized neonatal foals had inadequate delta-cortisol after a high-dose ACTH stimulation test, with the latter being significantly correlated with shock, multiple organ dysfunction (MODS) and decreased survival in one subset of septic foals. A low delta-cortisol response to a low-dose ACTH stimulation test was correlated with an increased risk of MODS but not shock (Hart *et al.*, 2009b). Overall, we found that horses with an ischemic gastrointestinal lesion had a poor response to a low-dose ACTH stimulation test, compared to those without an ischemic lesion. An inappropriately low delta-cortisol using a cut-off value < 5.65 µg/dL was identified in 81% of horses, while the more conservative cut-off of < 4.15 µg/dL was identified 67% of horses, with 50% at admission, 26% on day 2, 23% on day 4 and 22% on day 6. It is unknown which of these horses would have benefited from glucocorticoid therapy to moderate inflammatory responses or assist in blood pressure regulation.

5.5.7 Diagnosis of RAI/CIRCI in critically ill horses

When combined with clinical judgement of severity of illness in addition to either a low basal cortisol or low delta-cortisol (using the most conservative cut-off values), there were 12 horses ultimately diagnosed with RAI/CIRCI and only 10 survived. It is interesting that the two surviving cases were both colitis cases with SIRS and they both had basal cortisol concentrations lower-than-expected for illness or even lower than normal horses on all 4 test days. However, when low-dose ACTH stimulation tests were performed, they both had adequate delta-cortisol concentrations. In contrast, of the 10 horses that were euthanized due to a variety of severe conditions and were diagnosed with RAI/CIRCI due to an inadequate response to the low-dose ACTH stimulation test on at least on occasion, only 2 of these had low basal cortisol concentrations. Six of these cases were euthanized within the initial 24 hours and therefore endocrine testing was performed only once. Of the 3 non-surviving cases that had endocrine testing performed on 3 or 4 occasions, two of these had an inadequate response to ACTH stimulation on multiple occasions. Endocrine testing on day 2 of hospitalisation would eliminate having to test horses with rapidly fatal conditions and remove the influences of transport, sedation and dehydration, but vast numbers of critically ill horses would need to be enrolled to make any definitive recommendations as to the usefulness of performing ACTH stimulation and to refine appropriate cut-off values to diagnose RAI/CIRCI in critically ill adult horses.

5.5.8 Hypotension in CIRCI patients and considerations in our horses

Although not all human patients with septic shock have CIRCI, arterial hypotension refractory to fluid resuscitation and vasopressor support is one potential marker for inadequate glucocorticoid activity (Annane *et al.*, 2018). Many of our patients were likely experiencing hypovolemic shock, and some septic shock, but as assessment of blood pressure was not routinely performed, it was impossible to quantify in this study. Within 10 minutes of hospital admission clinically dehydrated animals would have been receiving high volume intravenous fluid therapy, but none were administered vasopressors prior to or after anesthesia. Administration of low dosages of glucocorticoids results in improvement of blood pressure in many cases of CIRCI, but also in non-CIRCI patients with septic shock, however patients with CIRCI had a poorer prognosis (Annane *et al.*, 2018; de Jong *et al.*, 2015; Sprung *et al.*, 2008). Due to the large numbers of patients required to determine the usefulness of low-dose glucocorticoid therapy and relevance to endocrinological test results in human medicine, and the conflicting results obtained between studies (Annane *et al.*, 2018), it is unlikely that such a study with sufficient power could be undertaken in critically ill horses to definitively prove the usefulness of glucocorticoids to help improve hypotension in standing or anaesthetized critically ill adult horses.

5.5.9 Glucocorticoid administration to CIRCI patients, side effects and the necessity for endocrinological testing

Inadequate cortisol mediated anti-inflammatory activity for the severity of illness is considered a defining feature of CIRCI. Meta-analysis of 27 randomized clinical trials with 3176 patients with sepsis (with and without shock), found a higher 28-day mortality (31.8%) in patients receiving a placebo compared with patients receiving corticosteroids (29.3%), with a relative risk of 0.87 [95% CI = 0.76 – 1.0] (Gibbison *et al.*, 2017). There was no increase in the rate of secondary infections and the most common adverse event was hyperglycaemia (Gibbison *et al.*, 2017). In human medicine the current recommendation is to administer low-dose corticosteroids to hospitalized patients with septic shock that are not responsive to fluid therapy and moderate to high-dose vasopressor therapy, but not to patients with sepsis alone (Annane *et al.*, 2018). As endocrinological testing has been shown to have a poor sensitivity and specificity for determining which patients will benefit from low-dose glucocorticoid administration, the recommendation is to treat based on clinical judgement rather than to await results of endocinological testing (Annane *et al.*, 2018; Sprung *et al.*, 2008) Prior to the commencement of this body of work, the recommendation was to perform ACTH stimulation testing on patients and then immediately commence glucocorticoid therapy if clinically indicated and then discontinue glucocorticoid therapy if results of endocrinological testing were normal (Annane, 2005). Treatment with low-

dose glucocorticoids in critically ill horses should be based on endocrinological testing until more information becomes available about the response to treatment in this group of patients. Eventually a large randomized, blinded, multicenter study would be required to determine if horses with or without CIRCI (based on inappropriately low basal or delta corticol concentrations) had differences in responses (positive and negative) after treatment with low-dose glucocorticoids.

5.5.10 Potential use and risks of glucocorticoids in horses with CIRCI

Although there has been no deleterious effects of low or moderate dose glucocorticoid therapy in human patients with septic shock, it is still unknown if empiric low-dose corticosteroid therapy would be useful or detrimental in critically ill horses. The specific criteria for recommendation of glucocorticoid therapy in critically ill horses is still uncertain, but could be possibly be considered in those with SIRS and MODs and certainly in those with unresponsive hypotension. However these horses are also at risk of laminitis (Parsons *et al.*, 2007). The relationship with prior administration of glucocorticoids and laminitis development is still hotly debated (Cornelisse & Robinson, 2013; Bailey & Elliott, 2007; Dutton, 2007; Johnson *et al.*, 2002). Although there are anecdotal results of laminitis occurring after administration of glucocorticoids in critically ill horses (Dr. Sally Church BVSc, FANZCVS, 1997) there are no published studies. Certainly there is a higher risk of laminitis with high dosages and prolonged courses of corticosteroids especially triamcinolone, potentially because of its induction of prolonged hyperglycaemia and hyperinsulinaemia (French *et al.*, 2000).

In a study involving 416 horses treated with oral prednisolone compared to two time-matched controls per case, there was no significant difference between the overall laminitis incidence rate, the rate of occurrence of laminitis whilst receiving prednisolone, or the probability of laminitis between the two groups, therefore the overall conclusion was that the use of oral prednisolone did not increase the risk of developing laminitis (Jordan *et al.*, 2017). There is no evidence to support high dose glucocorticoid therapy in critically ill patients that are not requiring immunosuppressive therapy and the doses of glucocorticoids that would be recommended in critically ill patients are much less than are routinely used for immunosuppressive or anti-inflammatory effects. In human medicine several different protocols for glucocorticoid administration to treat CIRCI have been recommended. The latest Cochrane systematic review of low-dose hydrocortisone for the treatment of septic shock (4268 patients) showed that corticosteroids reduced the risk of death at 28 days compared to a placebo. The study showed that survival benefits were better with lower doses of hydrocortisone (< 400 mg/day) for a longer duration (at least 3 days). Side effects were hyperglycaemia and hypernatremia, but there was no increased risk of gastrointestinal bleeding (Annane *et al.*, 2015). Administration of a continuous infusion of 200 mg of hydrocortisone/day for 5

days, followed by a tapering dose until day 11 is one such protocol (Keh *et al.*, 2016). This would equate to 0.2 mg/kg/day (based on an average 70 kg person). However this is still a supraphysiologic dose and results in serum cortisol concentrations several times higher than that achieved by ACTH stimulation. This dose was initially based on the maximum secretory rate of cortisol detected in humans following major surgery. The daily cortisol production rate and the pharmacokinetics of hydrocortisone have been studied in healthy neonatal foals and adult horses to determine the requirement of cortisol in healthy animals as a way to determine an appropriate dosage of hydrocortisone for critically ill horses (Hart, 2012). As a comparison, prednisolone has a glucocorticoid potency 4 times that of hydrocortisone and dexamethasone has a potency of 30 times that of hydrocortisone (Cuming *et al.*, 2018). Therefore at a dose of 0.2 mg/kg/day of hydrocortisone a 500 kg dose would receive 100 mg of hydrocortisone/day, 25 mg of prednisolone/day or 3.3 mg/day of dexamethasone as CRI administered over a 24 hr period.

5.5.11 Potential risk of hyperglycaemia with glucocorticoid therapy in horses with CIRCI

Although there has been no serious deleterious effects of low or moderate dose of hydrocortisone in septic humans, hyperglycaemia was one of the observed consequences (Keh *et al.*, 2016). As glucocorticoids result in elevations of serum glucose and insulin resistance, if glucocorticoids were to be administered to critically ill horses, then glucose and insulin concentrations should also be assessed, as pancreatic failure with relative hypoinsulinemia and hyperglycaemia has been detected in horses with SIRS and is associated with a poor prognosis (Bertin *et al.*, 2018a) paper III. Experimentally induced hyperglycaemia resulted in hyperinsulinemia and subclinical laminitis (de Laat *et al.*, 2012). Therefore concurrent monitoring of serum glucose and insulin concentrations and potentially even instigation of an insulin infusion to control hyperglycaemia would be advised if low-dose glucocorticoid therapy was instigated to treat RAI/CIRCI in critically ill horses.

5.5.12 Limitations of this study and further work

Major limitations of the study in addition to the lack of blood pressure assessment on all cases was the lack of dynamic testing to rule out PPID after patient recovery and the absence of histopathological examination of the adrenal glands of all deceased horses. Unfortunately adrenal gland autolysis occurs rapidly and necropsies typically occurred until 12-36 hours after death, therefore no useful correlations could be made between the presence of adrenal gland hemorrhage and necrosis and endocrinological test results. Further work includes correlation of direct blood pressure measurements, inflammatory mediators and histopathological assessment of the pituitary and adrenal glands of critically ill horses in relation to endocrinological test results. This may

allow specific determination of what an inadequate adrenal response to illness is and refinement of cut-off values for inadequate basal and delta-cortisol concentrations. Multicentre, randomized, placebo controlled clinical trials of the low of low-dose glucocorticoid therapy in horses diagnosed with CIRCI and any sick horses with inappropriately low basal or delta cortisol concentrations is warranted, but practically speaking it would be difficult to obtain sufficient numbers to achieve sufficient power without involving many referral hospitals.

5.5.13 Summary of findings of project V

Although high basal ACTH and cortisol concentrations were statistically higher at admission in non-survivors than survivors, and horses with ischemic gastrointestinal lesions had higher basal cortisol concentrations at admission than those without ischemic lesions, those with ischemic lesions failed to respond to a low-dose ACTH stimulation test. There was a large proportion of sick horses that had basal and delta-cortisol concentrations lower than those of normal horses indicating adrenal dysfunction. A subset of 12 horses that were identified as having adrenal dysfunction through endocrinological testing were ultimately diagnosed with adrenal insufficiency (RAI/CIRCI) based on their severity of illness, and these cases had a poor prognosis for survival. Of the two surviving cases and the high proportion of cases that had inappropriately low basal and ACTH-stimulated cortisol concentrations it is still unknown if they would have benefited from glucocorticoid administration.

6 Concluding remarks

This thesis contributes to the knowledge about testing adrenal function in foals and adult horses, in addition to the pituitary, adrenal and pancreatic responses to illness in hospitalized adult horses. The following specific conclusions can be drawn from the present work:

- In healthy adult horses, administration of 0.02, 0.1, 0.25 and 0.5 μg/kg of cosyntropin resulted in a significant increase in serum cortisol concentration for all 4 doses, compared with the baseline value, by 30 minutes after administration of cosyntropin.

- In healthy adult horses, administration of cosyntropin at a dose of 0.1 μg/kg (low-dose ACTH stimulation) resulted in maximum adrenal stimulation, with peak cortisol concentrations after 30 minutes.

- In healthy neonatal foals, administration of 0.1, 0.25 and 0.5 μg/kg of cosyntropin (but not 0.02 μg/kg) resulted in a significant increase in serum cortisol concentration, compared with the baseline value, by 10 minutes after administration of cosyntropin.

- In healthy neonatal foals, there was no difference in the peak cortisol concentrations obtained after administration of 0.25 and 0.5 μg/kg of cosyntropin at 20 and 30 minutes, respectively.

- In healthy adult horses, cosyntropin administration significantly increased the WBC and neutrophil counts and decreased the eosinophil count and the neutrophil-to-lymphocyte ratio. There was a doubling of the neutrophil/lymphocyte ratio compared with baseline, 240 minutes after administration of 0.25 or 0.5 μg/kg cosyntropin.

- In healthy neonatal foals, cosyntropin administration significantly decreased the lymphocyte count and increased the neutrophil/lymphocyte ratio. The neutrophil/lymphocyte ratio increased 2.4-fold at 120 minutes after administration of 0.25 µg/kg of cosyntropin.

- Hyperinsulinemia and hyperglycaemia are common problems of horses with SIRS; however, horses presenting with hyperglycaemia and corresponding relative hypoinsulinemia (suggestive of endocrine pancreas dysfunction) have a worse prognosis.

- A continuous rate infusion of insulin should be considered as a treatment for horses with hyperglycaemia and pancreatic dysfunction, with monitoring of serum glucose and insulin concentrations to ensure that hypoglycaemia or hyperinsulinemia does not occur.

- In sick horses, cortisol, ACTH and ACTH/cortisol ratios were higher in non-survivors at admission. ACTH/cortisol ratios were higher in horses with SIRS and those with an ischemic lesion, suggesting adrenal dysfunction.

- Although high basal ACTH and cortisol concentrations were statistically higher at admission in non-survivors than survivors, and horses with ischemic gastrointestinal lesions had higher basal cortisol concentrations at admission than those without ischemic lesions, those with ischemic lesions failed to respond to a low-dose ACTH stimulation test.

- A large proportion of sick horses had basal and delta-cortisol concentrations lower than those of normal horses indicating adrenal dysfunction.

- Adrenal insufficiency/CIRCI was diagnosed in 21% of sick horses based on endocrinological testing and the presence of critical illness and these cases had a poor prognosis for survival.

- It is unknown if the treatment with low doses of hydrocortisone would improve survival rates in horses diagnosed with CIRCI, but treatment trials are warranted in critically ill horses.

- It is unknown if treatment with low doses of hydrocortisone would improve outcome (survival or length of hospitalisation) in the high proportion of cases that had inappropriately low basal and ACTH-stimulated cortisol concentrations. Further work using clinical trials is required to further define cut-off values for these parameters in sick horses and determine whether glucocorticoid therapy to at least normalize cortisol concentrations is beneficial.

- If glucocorticoid therapy is instigated in the treatment of horses with inappropriately low basal or ACTH stimulated cortisol concentrations, then it will be important to monitor the effects on the glucose/insulin dynamics as concurrent insulin infusions may be required.

7 Future considerations

7.1.1 Cosyntropin pharmacokinetics

The radioimmunoassay used to measure ACTH concentrations does not differentiate endogenous ACTH concentrations from exogenous ACTH concentrations (from the administration of synthetic ACTH = cosyntropin). The ACTH stimulation test is a pharmacodynamics test of cosyntropin measuring the duration of action of the cosyntropin by measuring cortisol concentrations over time. It is not possible to determine the exact duration of persistence of cosyntropin product, but we have analysed ACTH concentrations after the administration of the 0.25 µg/kg dosage of cosyntropin administered to the 8 adult horses at each of the time points (T = 0, 30, 60, 90, 120, 180, and 240 minutes) after injection of cosyntropin and to the 11 neonatal foals at same time points and also T = 10 and 20 minutes in 6 of the foals. The ACTH measured will be a combination of exogenous and endogenous ACTH concentrations but some useful information on the half-life and clearance of cosyntropin may be able to be extracted. We know from our pharmacodynamics work in papers I and II that there is some negative feedback on cortisol concentrations occurring by 240 minutes which will be a confounding factor

7.1.2 Multivariate logistic regression analysis of factors associated with survival including glucose, insulin, ACTH and cortisol

The data set of 150 horses and over 40 variables is available for further potential analysis

7.1.3 Immunoreactive ACTH and stability issues

Liquid chromatography and high-resolution/high-accuracy mass spectrometry (LC-MS) are the gold standards to measure hormone concentrations. Although useful in a research setting LC-MS is not utilized for clinical cases due to the requirement of sophisticated equipment and specialised technical skills and the time involved in sample analysis. As there are no equine specific cortisol, ACTH or insulin standards or antibodies, all commercially available assays will likely underestimate equine hormones compared to LC-MC. The Scantibodies™ immunoradiometric kit used in the present study has been shown to be highly accurate, but measures 3.6-fold lower than the chemiluminescent Immulite™ (Schott, 2013). There is speculation that the ACTH measured by the Immulite™ may not all be $ACTH_{1\text{-}39}$ as the two-site antibody test that was designed and optimised for human ACTH may be cross reacting with other equine proopiomelanocortin derived peptides. A study using LC-MS to compare results obtained with the Immulite™ and other commonly used analysers and ACTH kits using equine plasma from normal horses, stressed horses and ACTH from horses with PPID pre and post thyrotropin releasing hormone stimulation should be performed and we are attempting to gain funding for such a study. This may explain the lower values obtained from the radioimmunoassays used in the current study compared to results obtained in other studies using the Siemens Immulite™.

As radioimmunoassays are becoming less available, the Siemens Immulite™ analyzer is being frequently used to measure equine ACTH. Recent work from our laboratory has recommended that when using this analyzer that plasma samples are not frozen, kept at 4°C, centrifuged within 2 hours and analyzed within 8 hours of collection to prevent misdiagnosis of PPID (Bertin *et al.*, 2018b). Less than ideal sample handling resulted in decreases and increases in ACTH concentrations and thus false positive and false negative diagnoses of PPID. Although this may not be relevant for stress induced ACTH originating from the pars distalis, the instability of immunoreactive ACTH appeared to be worse in horses with PPID compared to normal horses. As these ideal conditions of sample handling can not be readily achieved in equine general practice, accurate measurement of equine ACTH may be relegated to a teaching hospital.

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Popular science summary

In response to stress from exercise or illness, adrenocorticotropic hormone (ACTH) is released from the pituitary gland and acts on the adrenal glands to increase the production and release of cortisol. Cortisol is important in sick patients to increase the availability of glucose for energy production; moderation of the immune system and also in the regulation of blood pressure. In critical illness the concentrations of ACTH and cortisol should be very high, therefore it is expected that non-surviving patients may have higher concentrations of ACTH and cortisol than surviving patients with less severe disease. However the adrenal gland can be damaged or become "exhausted" during illness and production of cortisol may be much lower than expected for the degree of illness. Therefore inappropriately low cortisol concentrations are also associated with non-survival in critically ill patients. In surviving patients this adrenal dysfunction is transient and resolved after resolution of the underlying illness. This condition is known as relative adrenal insufficiency (RAI), which is part f the larger syndrome of critical illness related corticosteroid insufficiency (CIRCI). The diagnosis of RAI is made based on an inappropriately low cortisol concentration or an inadequate adrenal production of cortisol in response to administration of synthetic ACTH. Diagnosis and treatment of RAI/CIRCI results in an increase in survival rates in critically ill humans.

Investigation of appropriate dosages of synthetic ACTH to diagnose RAI in adult horses, or even if the condition existed in critically ill horses was previously unknown. We determined the most appropriate dosage of synthetic ACTH to cause adrenal gland stimulation and release of cortisol in healthy adult horses and neonatal foals.

We then measured ACTH and cortisol concentrations in 151 sick horses and administered synthetic ACTH to test for adrenal dysfunction in 58 sick horses. As expected, we found that sick non-surviving horses had higher concentrations of ACTH and cortisol than surviving horses at hospital admission. However we also found that 45% of sick horses had low cortisol concentrations at some time

during the initial 6 days of hospitalisation. We also found that 67% of the sick horses had an inappropriately low cortisol response after the administration of synthetic ACTH at some time during the initial 6 days of hospitalisation. These findings indicate adrenal dysfunction in sick horses. Adrenal dysfunction was identified in 21% of the critically ill horses, and only 2 of these 12 horses survived. These horses were considered to have RAI/CIRCI. Many of the non-surviving horses were euthanised due to severe intestinal damage that was not considered amenable to surgical repair. Treatment with low dosages of cortisone may be indicated in some critically ill and even some less severely sick horses to return concentrations of cortisol to at least that of normal horses when adrenal dysfunction is diagnosed. Further research is required to determine if such treatment will increase survival rates in critically ill horses as has occurred in critically ill humans.

During illness, as a consequence of the stress response, cortisol and other hormones increase the availability of glucose in the blood to provide energy for the patient. Excessive glucose concentrations are reduced by the production of the hormone insulin by the pancreas. Failure to produce sufficient insulin with the development of abnormally high blood glucose concentrations results in diabetes. Insufficient insulin concentrations and the development of transient or permanent diabetes is a rare diagnosis in adult horses. Although never reported in critically ill adult horses, diabetes has been reported in one critically ill foal.

Non-surviving sick horses often have markedly higher blood glucose concentrations than surviving horses, but insulin concentration had never been measured in sick horses prior to this work. We measured blood glucose and insulin concentrations in 51 horses with severe illness. Non surviving horses had higher glucose concentrations and lower insulin concentrations than surviving horses. This suggests that there is pancreatic dysfunction and a form of diabetes in critically ill horses.

Excessively high blood insulin or glucose concentrations have experimentally been shown to result in the development of laminitis, which is an exceeding painful condition where the sensitive and non-sensitive structures of the hoof separate. Laminitis also occurs in critically ill horses and can be so severe that the horse must be euthanised due to the laminitis even if the underlying primary disease or condition has been successfully medically or surgically resolved. In critically ill humans, regulation of blood glucose concentrations by the administration of insulin has improved survival rates in some studies. Administration of insulin to sick horses that have high blood glucose and low serum insulin may be advantageous and may even prevent laminitis in some cases. Further research is required to confirm that such treatment improves survival in critically ill horses.

Populärvetenskaplig sammanfattning

Fysiskt arbete eller sjukdom ger upphov till en stressrespons i kroppen. Adrenokortikotropt hormon (ACTH) släpps ut från hypofysen och påverkar binjuren vilket i sin tur ökar produktionen av kortisol. Kortisol har en viktig roll vid sjukdom för att öka tillgången på glukos för energi, samt för att moderera immunsvaret och reglera blodtryck. Vid allvarlig sjukdom stiger ACTH- och kortisolkoncentrationen kraftigt och därför borde de sjukaste, icke-överlevande, patienterna ha högre halter av dessa ämnen än de som tillfrisknar igen. Det är dock så att binjuren kan bli ”utmattad” under sjukdomsprocessen och kortisolhalterna kan därför vara mycket lägre än förväntat med tanke på sjukdomens allvarlighetsgrad. Låga kortisolnivåer är därför också associerat med sämre överlevnad hos intensivvårdspatienter. Hos överlevande är binjureinsufficiensen övergående och försvinner i samband med att den underliggande sjukdomen avtar. Tillståndet kallas ”relative adrenal insufficiency” (RAI), och är en del av det mer omfattande tillståndet sjukdomsrelaterad sekundär kortisolsvikt (critical illness related corticosteroid insufficiency, CIRCI). Diagnosen RAI ställs baserat på onormalt låga kortisolnivåer vid sjukdom, eller genom att mäta responsen efter injektion av syntetiskt ACTH. Diagnos och behandling av RAI/CIRCI ökar chansen för överlevnad inom humanvården.

På hästsidan har vi tidigare saknat information om ifall samma sjukdomstillstånd existerar, och hur man i så fall bäst doserar ACTH för testning.

Vi fastställde lämplig dos syntetiskt ACTH för binjurestimulering och kortisolfrisättning hos friska hästar och neonatala föl. Därefter analyserades ACTH och kortisolnivåer hos 151 sjuka hästar. Sjuka hästar som inte överlevde hade högre nivåer av ACTH och kortisol vid ankomst till klinik än de som klarade av sjukdomen och överlevde. Vi fann dock att 45 % av de sjuka hästarna hade oväntat låga kortisolnivåer någon gång under de första 6 dagarna av sjukhusvistelsen. Syntetiskt ACTH gavs till 58 hästar för att testa binjurefunktionen. Vi upptäckte då att 68 % av dessa hade för dålig kortisolrespons efter injektion av syntetiskt ACTH vid något tillfälle under de 6 första dagarna. Detta indikerar att binjureinsufficiens förekommer hos sjuka

hästar. Uttalad binjureinsufficiens (RAI/CIRCI) identifierades hos 21% av hästarna som intensivvårdades, och endast 2 av dessa 12 hästar överlevde. Många av de hästar som inte klarade sig avlivades p.g.a. grava tarmskador som bedömdes inoperabla.

Behandling med låg dos kortison kan vara indicerat för en del intensivvårdspatienter och även för vissa mindre kritiskt sjuka hästar för att återställa kortisolnivåerna när binjureinsufficiens konstaterats. Fler studier behövs för att fastställa om sådan behandling kan förbättra överlevnaden för akut sjuka hästar på samma sätt som det gjort på humansidan.

Vid sjukdom bidrar stressresponsen till att kortisol och andra hormon frigör mer glukos för att bidra med energi. Insulin frisätts från bukspottkörteln för reglera att blodglukosnivåerna inte blir för höga. Om otillräcklig mängd insulin produceras, kommer detta att resultera i kraftigt förhöjda glukosnivåer och diabetes. Detta tillstånd är ovanligt och har aldrig rapporterats hos akut sjuka vuxna hästar, men har rapporterats hos ett kritiskt sjukt föl.

Icke-överlevande sjuka hästar har ofta markant högre blodglukosnivåer än överlevande, men insulinnivåer har inte tidigare utvärderats hos sjuka hästar. Vi analyserade blodglukos och insulinnivåer hos 51 gravt sjuka hästar. Icke-överlevande hade högre glukoskoncentrationer och lägre insulinnivåer än överlevande hästar. Detta indikerar att riktigt sjuka hästar kan ha nedsatt funktion av bukspottkörteln och i och med detta en typ av diabetes.

Överdrivet höga halter av insulin eller glukos i blodet har experimentellt visats kunna leda till fång, en mycket smärtsam sjukdom med separation av lamellerna i hovväggen. Fång är också en allvarlig komplikation hos kritiskt sjuka hästar och kan i vissa fall bli så problematisk att den i sig leder till avlivning trots att den underliggande sjukdomen kunnat behandlas. Inom humanvården har man i vissa studier visat på förbättrad överlevnad när man aktivt kontrollerat blodglukosnivåer genom att tillföra insulin. Det kan vara så att tillförsel av insulin till hästar med förhöjt blodglukos skulle kunna vara fördelaktigt och förebygga fång i vissa fall. Vidare studier krävs för att konfirmera om sådan behandling skulle förbättra överlevnaden hos häst.

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Appendix 1. Information for clients regarding study enrolment

Dear horse owner,

As part of a study to eventually help critically ill horses we are measuring some of the naturally released stress hormones that are released in times of illness. During stressful events (exercise, transportation and illness) the body increases its production of the hormones cortisol and ACTH (the substance that causes the release of cortisol). Information from human intensive care patients indicates that during times of very severe illness, the body's supply of natural cortisol and ACTH is inappropriately low. It appears that supplemental therapy to supply cortisol improves survival rates for critically ill humans.

Unfortunately, little is known about the effects of severe illness on cortisol and ACTH concentrations and release in horses. Recognition of inadequate cortisol and ACTH release may help identify individual horses that may benefit from low-level cortisol treatment.

At Auburn University, we are proud to be at the forefront of equine intensive care. We wish to gather information about cortisol and ACTH levels in the sickest horses that are treated at the John Thomas Vaughan Large Animal Clinic (JTVLAC). Publishing our findings may assist countless other critically ill horses worldwide.

We would like to offer your horse an increased level of intensive care monitoring by measuring his/her cortisol and ACTH concentrations. Unfortunately, results may not be immediately available, but the results from your horse may eventually help other horses.

We would like to collect an additional 27 mls of blood (at the same time that routine bloodwork is performed) and perform an ACTH stimulation test on four occasions. The ACTH stimulation test is extremely safe and is routinely performed in human intensive care patients. The ACTH test has been performed in normal and exercising horses and premature foals without any side-effects. The collection of the blood and the analysis of blood results will not affect the treatment that your horse will receive.

In order to collect blood and record your horse's results in our study, we must obtain your written permission. **The additional testing will be performed free of charge.** We hope to eventually publish the results of our study, but your name and your horse's name and details will remain confidential. The results of our study may enhance the care of future generations of equine patients.

If you would like your horse included in the study, please sign the following statement.

Appendix 2. Client consent form for use of client owned horses from Auburn University for Identification of Relative Adrenal Insufficiency in Critically Ill Adult Horses and Neonatal Foals

I _____give permission for an additional 27 mls of blood to be drawn from my horse at admission and then every 2 days on 3 more occasions, at the same time that routine bloodwork monitoring is performed during the period of hospitalisation. I also give permission for an ACTH stimulation test to be performed on 4 occasions, which involves the collection of an additional 10 mls of blood after the administration of a small dose of ACTH. I understand that the cost of ACTH is expensive, **but this additional testing will be performed free of charge**. This study will also pay for my routine bloodwork on 2 occasions, which will be discounted from my bill. I understand that this additional monitoring will in no way affect the quality or type of care my horse will receive. I also understand that to have been chosen to be included in this study that my horse is extremely sick, and I am responsible for the payment of the care that he/she requires regardless of the outcome.

Signed:_____Date:_____

Print:_____

Phone: _____Fax:_____

Horse's name:_____Case Number:_____

Age:_____ Breed: Sex:_____

My veterinarians' name:_____Phone: _____

Thank-you for your participation in this study. At Auburn University we are committed to offer the best in equine intensive care. We also hope that we can contribute scientific knowledge that may be of benefit to horses worldwide. Auburn University is committed to excellence in teaching, research and outreach, and at the JT Vaughan Large Animal Hospital faculty and staff will strive to provide the ultimate in intensive care,

Yours sincerely,

Allison Stewart BVSc(Hons), MS, Diplomate American College of Veterinary Internal Medicine, Fellow in Veterinary Emergency and Critical Car