Dissertation

THE ROLE OF INSULIN IN BLOOD GLUCOSE ABNORMALITIES IN

CANINE BABESIOSIS

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DECLARATION

I hereby declare that this dissertation, submitted for the MMedVet (Med) degree, to the University of Pretoria, is my own work and has not been submitted to another university for a degree, and that the data included in this dissertation are the results of my investigations.

Philip Rees

23 April 2010

DEDICATION

To my ever-loving and supportive wife Desiré, and my daughter Natalie.

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SUMMARY

Abnormal carbohydrate metabolism is a commonly encountered feature of malaria in people, and similar derangements have been detected in veterinary patients with canine babesiosis. Glucose, the major metabolic fuel source, is a key resource in critically ill patients as they mount an immunological response to infection and inflammation. The ability of the individual to effectively mobilise, distribute and utilise glucose is a major determinant of morbidity and mortality. Hypoglycaemia has been identified as a life threatening metabolic complication in almost 20% of severely ill dogs suffering from babesiosis due to *Babesia rossi* infection. Insulin and glucagon are the primary hormones involved in glucose homeostasis. Insulin lowers blood glucose concentration by facilitating cellular uptake and utilisation of glucose. Hyperinsulinaemia as a result of inappropriate insulin secretion may precipitate hypoglycaemia, and has been identified as a cause of hypoglycaemia in human and murine malaria. A similar phenomenon may exist in canine babesiosis.

This prospective, cross-sectional, observational study, including 94 dogs with naturally acquired virulent babesiosis, sought to investigate and characterise the relationship between blood glucose concentrations and insulin concentrations in cases of canine babesiosis. Pre-treatment jugular blood samples were collected for simultaneous determination of plasma glucose and insulin concentrations. Animals were retrospectively divided into three groups: hypoglycaemic (plasma glucose concentration < 3.3 mmol/L; n=16), normoglycaemic (3.3-5.5 mmol/L; n=62), and hyperglycaemic (> 5.5 mmol/L;

n=16). The median plasma insulin concentrations (IQR in parentheses) for the hypoglycaemic, normoglycaemic and hyperglycaemic groups were 10.7 pmol/L (10.7-18.8 pmol/L), 10.7 pmol/L (10.7-29.53 pmol/L; i.e below the detection limit of the assay), and 21.7 pmol/L (10.7-45.74 pmol/L), respectively. Statistical analysis revealed no significant difference in insulin concentration between the three groups. These results suggest that insulin secretion was appropriately suppressed in these dogs. Only two dogs had elevated insulin concentrations, one of which was hypoglycaemic. The median time since last meal (available for 87 dogs) was 24 hours (IQR 2-4 days), constituting a significant period of illness-induced starvation.

We conclude that hyperinsulinaemia is not a cause of hypoglycaemia in virulent canine babesiosis. It is speculated that prolonged fasting due to disease-induced anorexia, in addition to increased glucose consumption, depletion of hepatic glycogen stores, and hepatic dysfunction with impaired gluconeogenesis, may play important roles in the pathophysiology of hypoglycaemia in canine babesiosis.

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
g/L	grams per litre
GH	Growth hormone
GLUT	Glucose transporter
ICU	Intensive care unit
IDE	Insulin degrading enzyme
IL-1	Interleukin-1
IL-6	Interleukin-6
IPGs	Inositol phosphoglycans
IQR	Interquartile range
IRI	Immunoreactive insulin
ISA	In saline agglutination
LT	Lymphotoxin
M	Molar
mL	Millilitre
mmol/L	Millimoles per litre

MODS	Multiple organ dysfunction syndrome
mRNA	Messenger ribonucleic acid
NEM	*n*-ethylmaleimide
OVAH	Onderstepoort Veterinary Academic Hospital
PCR	Polymerase chain reaction
PCV	Packed cell volume
PEPCK	Phosphoenolpyruvate carboxykinase
pmol/L	picomoles per litre
RIA	Radioimmunoassay
RLB	Reverse line blot
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation
SIRS	Systemic inflammatory response syndrome
SSU	Small subunit
TNF	Tumour necrosis factor
TSP	Total serum protein
µL	microliter

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Derangements in carbohydrate metabolism manifesting as hyperglycaemia, hypoglycaemia, and hyperlactataemia, are common findings in canine babesiosis (Keller and others 2004; Nel and others 2004; Jacobson and Lobetti 2005). Hypoglycaemia and hyperlactataemia have been associated with an unfavourable outcome (Keller and others 2004; Nel and others 2004). Hyperglycaemia is an independent risk factor for morbidity and mortality in critically ill human intensive care unit (ICU) patients (Mizock 2001; Van den Berghe and others 2001). Similar increases in morbidity and mortality have been identified in hyperglycaemic critically ill dogs (Torre and others 2007). The likely causes of hyperlactataemia in canine babesiosis have been discussed elsewhere (Keller and others 2004; Nel and others 2004; Jacobson and Lobetti 2005), but the mechanisms leading to hyper- and hypoglycaemia are as yet unclear. These abnormalities may reflect underlying endocrine derangements, particularly in severe and complicated babesiosis.

Insulin is one of the major hormones regulating blood glucose concentration. It has been shown in murine and human malaria, a disease sharing many similarities with babesiosis, that hypoglycaemia may occur in association with hyperinsulinaemia (Elased and Playfair 1994). No information exists concerning the patterns of insulin secretion in critically ill dogs with babesiosis.

In this setting, hyperinsulinaemia may contribute to hypoglycaemia, and knowledge of this may lead researchers down new therapeutic avenues.

1.2 Carbohydrate metabolism

1.2.1 Overview

Carbohydrate metabolism depends on a complex system of enzymatic biochemical reactions aimed at the assimilation, distribution, storage and conversion of carbon fuel molecules to usable energy for cellular processes. This process is regulated very closely by an array of hormonal signals that produce wide-ranging effects in all tissues of the body. The basic carbohydrate unit is the hexose sugar glucose. This molecule is polymerised for storage in the liver and muscle as glycogen, and synthesised *de novo* during gluconeogenesis in the liver when glucose from food is scarce.

1.2.2 Glucose metabolism

Glucose is the major cellular carbohydrate fuel source and is metabolised in cells to provide adenosine triphosphate (ATP). This ubiquitous molecule is the 'energy currency' of the body. The oxidation of one molecule of glucose via the anaerobic glycolytic pathway and Krebs cycle produces 4 molecules of

ATP. During aerobic oxidative phosphorylation the process is much more efficient and the yield from each glucose unit is 32 molecules of ATP. The total ATP yield from one glucose molecule by the coupling of glycolysis, the Krebs cycle and oxidative phosphorylation is therefore 36-38 ATP molecules (Stryer 1988). Dietary glucose is derived from three main sources i.e. sucrose (disaccharide from cane sugar), lactose (disaccharide from milk) and starches (polysaccharides from many sources, especially grains). The enzymes sucrase, lactase, maltase and α-dextrinase that split disaccharides and small glucose polymers are located in the outer face of the plasma membrane of brush border microvilli of intestinal epithelial cells (Stryer 1988). Sucrose is thus hydrolysed to glucose and fructose by sucrase. Lactose is hydrolysed to the monosaccharide galactose by lactase. Starches are cleaved to form the disaccharide maltose by pancreatic amylase. Maltose and other small glucose polymers are hydrolysed by maltase and α-dextrinase to form glucose. These basic monosaccharides are absorbed by the intestinal microvilli and enter the portal circulation (Stryer 1988). Absorption of glucose in the small intestine is facilitated by sodium-glucose co-transporter carriers. This is an active transport process that moves glucose across the cell membrane in conjunction with sodium (Voet and others 2002). From the intestine, glucose is transported directly to the liver where it diffuses into the hepatocytes.

The movement of glucose into hepatocytes occurs via a process of facilitated diffusion, involving a molecule belonging to a family of glucose transporter molecules. At least five of these transporter isoforms have been described.

The first, glucose transporter 1 (GLUT1), is found in the blood brain barrier, placenta, and red blood cells, and has a high affinity for glucose, ensuring glucose uptake even under conditions of hypoglycaemia. GLUT2 is expressed in tissues such as liver, kidney, small intestine and pancreatic β-cells. In the liver, GLUT2 mediates the uptake and release of glucose by hepatocytes. In the pancreas, GLUT2 regulates glucose-stimulated insulin secretion. GLUT3 is present in the brain and nerves. Glucose transport by GLUT1, GLUT2 and GLUT3 is insulin-independent. GLUT4 is present only in tissues where glucose uptake is mediated by insulin i.e. muscle, adipose tissue and cardiac muscle (Mizock 2001; Voet and others 2002).

After diffusing into the hepatocyte cytoplasm (GLUT2-mediated), glucose is immediately phosphorylated by the enzyme glucokinase (hepatic isomer of hexokinase) to form glucose-6-phosphate. The phosphorylation of glucose in most tissues is almost irreversible and serves to capture glucose in the cell (Stryer 1988). The only exceptions are the liver parenchymal cells, renal tubular and intestinal epithelial cells that possess glucose phosphatase for reversing this reaction (Voet and others 2002).

Glucose-6-phosphate may be utilised directly for energy production by entering the glycolytic pathway, or may be stored as glycogen by the process of glycogenesis. Hepatic glycogen synthetase transfers glucose moieties to the growing glycogen chain in α-1, 4 linkages until the chain reaches 7-24 glucosyl units in length. Thereafter a branching enzyme adds α-1, 6 bonded

glucose subunits to sections of the glycogen molecule (Stryer 1988). Control of glycogen synthetase is partly achieved by alterations in intracellular cyclic adenosine monophosphate (cAMP) concentrations. Glycogen is stored in the liver as well as muscle cells. The liver stores approximately 50% of intestinally absorbed glucose by conversion to glycogen; the remainder is distributed to the extracellular space. Rising extracellular glucose concentrations result in entry of glucose into β-cells via GLUT2. Glucose then enters the glycolytic pathway within the β-cell and is oxidised to form ATP. ATP-controlled K^+ - channels close producing cell membrane depolarisation and the opening of voltage-controlled Ca^{2+}-channels. Intracellular Ca^{2+} concentrations rise, producing the release of stored insulin and C-peptide in equimolar amounts into circulation from secretory vesicles (Stryer 1988).

Hexokinase itself exists as four isoenzymes in mammals. These isoenzymes have differing affinities for glucose (Stryer 1988; Voet and others 2002). Hexokinase 1 is found in the brain, and has a very high affinity for glucose thus ensuring adequate metabolic substrate for the brain even when extracellular glucose concentrations are low. In the liver, however, the hexokinase isoenzyme (glucokinase) has a much lower affinity for glucose (about 200 times less than hexokinase I). This means that glucokinase functions to remove excess glucose from the blood when glucose concentrations rise above normal, such as post prandially.

During a short fast, endogenous glucose production from hepatic glycogenolysis and gluconeogenesis provides metabolic substrate for tissues

(Turnwald and Troy 1983). Beyond 24 to 48 hrs of fasting, gluconeogenesis is the major source of glucose as glycogen stores are depleted. During this time, glucagon, catecholamine and cortisol secretion predominate, promoting gluconeogenesis (Cryer and Polonsky 1998).

1.3 Endocrine control of glucose metabolism

1.3.1 Glucagon

Glucagon is a 29-residue polypeptide hormone synthesised by the α-cells of the pancreas. When glucose concentration falls, insulin concentrations are low, and glucagon is produced in the α-cells of the pancreas and secreted into the blood stream (Stryer 1988; Guyton and Hall 2000). The main target organ of glucagon is the liver. Glucagon promotes hepatic glycogenolysis and inhibits glycogen synthesis. The lowering of intracellular fructose 2, 6-bisphosphate concentration by glucagon inhibits glycolysis and stimulates gluconeogenesis. Glucagon also inhibits fatty acid synthesis. The actions of glucagon are mediated by protein kinases activated by cAMP (Mizock 2001). The combined cellular effects of glucagon result in a net increase in hepatic release of glucose and triacylglycerol release from adipose tissue. The secretion of glucagon (elicited by an increase in sympathetic nervous system tone) in response to falling blood glucose concentrations is rapid and has a

relatively short-lived effect (Guyton and Hall 2000). This also holds true for the catecholamines, discussed below.

1.3.2 Epinephrine and norepinephrine

Catecholamines increase the amount of glucose released into circulation by the liver and decrease muscle utilization of glucose (Cryer and Polonsky 1998). In response to low blood glucose concentrations, catecholamines are secreted by the adrenal medulla and sympathetic nerve endings and promote the mobilization of glycogen and triacylglycerols via the cAMP cascade. The glycogenolytic effect of catecholamines is greater in muscle than in the liver. They also promote the preferential use of fatty acids as a fuel source by muscle. The release of glucagon is stimulated and the secretion of insulin is inhibited (Stryer 1988; Cryer and Polonsky 1998).

1.3.3 Cortisol

The steroid hormones (progestagens, glucocorticoids, mineralocorticoids, androgens and oestrogens) are synthesised from cholesterol. The glucocorticoids, notably cortisol, are important hormones with multiple effects in the body. In contrast to hormones such as epinephrine and insulin, which bind to a cell surface receptor and exert their effect via second messenger

molecules, cortisol (hydrocortisone) enters the cell and binds to an intracellular receptor. This hormone-receptor complex is transported into the nucleus where it binds to specific sites on deoxyribonucleic acid (DNA) and enhances transcription (Guyton and Hall 2000; Voet and others 2002).

The systemic effects of cortisol are wide-ranging. Protein metabolism is driven into a catabolic state, with a reduction in muscle protein synthesis and mobilisation of amino acids. Fatty acids are mobilised from adipose tissue. Carbohydrate metabolism is significantly altered by cortisol. Following DNA transcription and messenger ribonucleic acid (mRNA) synthesis, gluconeogenic enzymes are synthesised. This increase in gluconeogenesis by increasing concentrations of the pathway enzymes is the major effect of cortisol. Cortisol also decreases the rate of glucose utilisation by cells. Consequently, the overall effect of cortisol is to raise blood glucose concentrations (Stryer 1988; Cryer and Polonsky 1998).

Cortisol secretion in response to hypoglycaemia is a delayed response, but its effects persist for 4-6 hours (Leifer and Peterson 1984; Walters and Drobatz 1992). Cortisol is an important hormone, vital in normal homeostasis as well as the metabolic response to stress of any nature, including infection, trauma, starvation and mental stress. Cortisol stabilises intracellular lysosomal membranes, thus exerting a potent anti-inflammatory effect.

1.3.4 Growth hormone

The peptide hormone growth hormone (somatotropin) is produced in the anterior pituitary (adenohypophysis), and, like cortisol, exerts a delayed but persistent effect on blood glucose concentrations. Growth hormone (GH) secretion results in decreased peripheral glucose utilisation and increased hepatic gluconeogenesis. These effects combine to increase blood glucose concentrations (Guyton and Hall 2000).

1.3.5 Insulin

Insulin is secreted in response to rising blood glucose concentrations and stimulation of the β-cells of the pancreas by the parasympathetic nervous system. Insulin stimulates anabolic processes and inhibits catabolic processes. It is the predominant hormone (along with glucagon) in the control of blood glucose concentrations. It is secreted as a single polypeptide prohormone (called proinsulin) by the β-cells of the pancreatic islets of Langerhans and released into the bloodstream after hydrolysis of the connecting peptide (C-peptide) in response to rising glucose concentrations. The active hormone exists as a dipeptide (A and B chain) connected by two disulphide bonds (Stryer 1988; Guyton and Hall 2000; Voet and others 2002).

The insulin receptor of target organs is a trans-membrane protein. The receptor occurs at very low densities on the plasma membrane of target cells (about one receptor per square micrometer of plasma membrane of adipose cells) but has a very high affinity for insulin. This is necessarily so since insulin concentrations in blood are very low. The binding of insulin to its receptor switches on the intracellular tyrosine kinase activity of the receptor, phosphorylating target intracellular proteins (Voet and others 2002).

Insulin stimulates synthesis of glycogen (in the liver and muscle), proteins (in muscle) and lipids (in adipose tissue and liver) by promoting intracellular uptake and utilization of glucose and amino acids (Stryer 1988). Glycolysis is stimulated, while glycogenolysis, lipolysis and gluconeogenesis are inhibited, as is protein degradation. During fasting, insulin concentrations are low while the counter-regulatory hormones glucagon, catecholamines and growth hormone promote hepatic glycogenolysis and gluconeogenesis from lactate (end product of glucose metabolism and Cori cycle), amino acids (especially alanine derived from skeletal muscle) and glycerol from triglyceride hydrolysis (Leifer and Peterson 1984). The supply of these non-glucose gluconeogenic precursors is the rate-limiting step for gluconeogenesis (Voet and others 2002).

1.4 Carbohydrate metabolism in disease

Significant alterations in carbohydrate metabolism occur in many disease scenarios, including infectious disease, sepsis, and neoplasia. During critical illness, energy expenditure is increased dramatically and in acute febrile disease cortisol secretion may be increased by up to six times (Chandler and others 1992).

1.4.1 Hyperglycaemia

The general causes for hyperglycaemia in dogs and cats are listed in Table 1. In critically ill patients, the following are potential causes of hyperglycaemia: increased catecholamine secretion (endogenous or exogenous), increased glucocorticoid secretion (endogenous or exogenous), insulin resistance, total parenteral nutrition, dextrose infusion, surgery, anaesthesia, and inflammatory mediators (Knieriem and others 2007). Hyperglycaemia is a common occurrence in human critical illness and has been associated with increased risk of morbidity and mortality (Capes and others 2000; Mizock 2001). This phenomenon of 'stress hyperglycaemia' and hypermetabolism is a response to severe injury or infection (Chandler and others 1992; Mizock 1995) and is akin to that seen in malaria (Krishna and others 1994) and babesiosis (Jacobson and Lobetti 2005). The syndrome manifests as hyperglycaemia, insulin resistance and protein catabolism. In a recent study (Keller and others 2004), fifteen percent of dogs with babesiosis were hyperglycaemic at presentation (as opposed to 9% that were hypoglycaemic). Despite its greater

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prevalence, hyperglycaemia was not a reliable indicator of disease severity. In another study of 20 dogs with babesiosis, 2 (10%) were hyperglycaemic, whereas hyperlactataemia was frequently present (Jacobson and Lobetti 2005).

Diabetes mellitus

Stress (especially cats)

Postprandial hyperglycaemia, especially with diets containing sugars

Dextrose infusion

Hyperadrenocorticism

Acromegaly (cat)

Dioestrus (bitch)

Phaeochromocytoma

Pancreatitis

Exocrine pancreatic neoplasia

Renal insufficiency

Drug therapy- glucocorticoids, progestagens, megestrol acetate

Parenteral nutrition

Cranial trauma

Table 1. Differential diagnosis of hyperglycaemia (Nelson 2005; Knieriem and others 2007)

The adverse effects of hyperglycaemia as reported by Mizock (Mizock 2001) include osmotic diuresis with resultant hypovolaemia, electrolyte abnormalities and hyperosmolar non-ketotic coma. Hyperglycaemia inhibits neutrophil function by impairing phagocytosis and diminishing production of oxygen radicals. Stimulation of coagulation (Bernard and others 2001) and modulation of endothelial function (Langouche and others 2005) have also been demonstrated. Strict glycaemic control in critically ill patients has been shown to significantly reduce morbidity and mortality (Van den Berghe and others 2001). Although no consensus has been reached concerning optimum target glucose values in these patients, intensive insulin therapy undoubtedly is a valuable tool in the management of critically ill human patients and may also have merit in veterinary patients (Knieriem and others 2007; Torre and others 2007), including cases of severe babesiosis with hyperglycaemia.

1.4.2 Hypoglycaemia

Euglycaemia represents a balance between production, storage, and release of glucose (Walters and Drobatz 1992). Plasma glucose and insulin concentrations have a direct inverse relationship. Rising concentrations of glucose in plasma stimulate the release of insulin into the circulation by the pancreatic islet β-cells. Normal glucose concentrations in the dog are 3.3-5.5 mmol/L while insulin concentrations fluctuate between 35-180 pmol/L (Reimers and others 1982; Parsons and others 2002). Insulin release is progressively inhibited as glucose concentration falls below 4.6 mmol/L (Cryer

and Polonsky 1998). Counter-regulatory hormones antagonising the effects of insulin include glucagon, catecholamines, growth hormone, and cortisol (Cryer and Polonsky 1998).

Hypoglycaemia is defined as a blood glucose concentration of less than 3.3 mmol/L (Feldman and Nelson 2004). However, the development of clinical hypoglycaemia is highly variable at a wide range of values below this limit. Whipple's triad has been used in human medicine to identify clinical hypoglycaemia. Three criteria must be satisfied namely: a blood glucose concentration below 2.7 mmol/L; simultaneous neuroglycopoenic symptoms; and relief of symptoms with correction of the low blood glucose concentration. Hypoglycaemia may result from increased glucose utilization (e.g. pancreatic β-cell tumours), decreased production of glucose (e.g. hepatic disease, hypoadrenocorticism), or a combination of these (e.g. hypermetabolism of sepsis). Signs of hypoglycaemia are usually attributable to cerebral dysfunction i.e. stupor, coma, seizures, behaviour changes, as well as muscle weakness, ataxia and collapse. Neurological signs predominate because the brain does not store glycogen and possesses a limited ability to utilize energy sources other than glucose (and ketones). Tissues such as peripheral nerves, renal and adrenal medullary cells, red and white blood cells, cardiac myocytes, and platelets are also able to utilize fatty acids and ketones as alternative energy sources.

The causes of hypoglycaemia are varied and result from disruption of glucose homeostatic mechanisms. The general causes for hypoglycaemia are listed in

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Table 2. The initial physiological response to early hypoglycaemia is stimulation of the sympathetic nervous system resulting in the release of epinephrine and glucagon, which have a short-lived but immediate effect. They raise blood glucose by inhibiting peripheral glucose utilization, increasing hepatic glycogenolysis and gluconeogenesis and inhibiting insulin secretion. Cortisol and growth hormone decrease peripheral glucose utilization and increase hepatic gluconeogenesis. Their release is delayed by a few hours, but their effects persist for four to six hours (Leifer and Peterson 1984; Walters and Drobatz 1992).

Neonates and toy breeds (younger than six months of age)
Glycogen storage diseases, other enzyme defects
Drug-induced causes (ethanol, insulin, sulfonylureas)
Hyperinsulinaemia (insulinoma)
Paraneoplastic causes
Extrapancreatic neoplasia
Hepatic disease (impaired gluconeogenesis)
Hypoglycaemia associated with cardiac disease and congestive heart failure
Renal failure
Adrenocortical insufficiency (Addison's disease)
Endotoxaemia/ Sepsis
Canine Babesiosis
Hypopituitarism

Pregnancy-associated hypoglycaemia/ ketonuria

Exercise-induced hypoglycaemia (hunting dogs)

Malnutrition/Starvation

Xylitol intoxication

Table 2. Differential diagnosis of hypoglycaemia (Drobatz and Mandell 2000; Hess 2005)

Sepsis

Sepsis is an important cause of morbidity and mortality in human critical care settings (Hinshaw and others 1977). In this setting, blood glucose abnormalities are common. Following the early hypometabolic ebb phase (which may not occur in sepsis, but occurs in trauma and burns), the hypermetabolic flow phase peaks at 3-4 days and then slowly abates (Cerra 1987). This phase is characterised by raised absolute insulin concentrations, yet an increased glucagon/insulin ratio is present, resulting in increased gluconeogenesis and hyperglycaemia (Cerra 1987; Mizock 2001) along with signs of systemic inflammation. Increased gluconeogenesis is also associated with increased whole-body glucose uptake and insulin resistance. Cytokines may also play a role here by inhibiting insulin release (tumour necrosis factor [TNF], interferon-α) or by stimulating the hypothalamic-pituitary-adrenal axis in

promoting the release of corticotropin-releasing hormone and adrenocorticotropic hormone (TNF, interleukin [IL]-1) (Mizock 2001).

Hypoglycaemia may also manifest during sepsis. A biphasic response has been noted in lethal models of sepsis, characterised by early hyperglycaemia (increased gluconeogenesis) followed by subsequent hypoglycaemia (suppressed glucose production) (Mizock 1995). Mechanisms underlying hypoglycaemia in sepsis include altered microcirculatory blood flow, impaired glucose production (due to depressed hepatic function and gluconeogenesis as organ failure sets in) (Woolf and others 1979), abnormal glucose uptake or utilization, and insulin or insulin-like influences (Breitschwerdt and others 1981). One suggested mechanism of this impairment is the suppression of hepatic gluconeogenesis by insulin and cytokine-mediated reduction in expression of the phosphoenolpyruvate carboxykinase (PEPCK) gene (Deutschman and others 1993). This is important, because the enzymatic activity of PEPCK (and thus the rate of gluconeogenesis) is directly determined by the absolute number of molecules of the enzyme in the cell.

Studies of endotoxic shock in dogs showed a marked hyperinsulinaemia in dogs pre-treated with intravenous glucose (Blackard and others 1976). These trials failed to demonstrate hyperinsulinaemia when glucose was not administered, suggesting that hyperinsulinism is unlikely to be the cause of hypoglycaemia in endotoxic states. Rather, they support the notion that the stimulatory effects of blood glucose on pancreatic insulin secretion are

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potentiated in endotoxic states. Alternatively, in sepsis-associated hyperglycaemia, the glucose-lowering effects of insulin may be impaired, as seen in diabetic ketoacidosis associated with infection.

The white cell response to endotoxin shock, namely utilization of the glycolytic pathway during increased phagocytic activity, may account (at least in part) for increases in the utilization of glucose (and subsequent hypoglycaemia) by the blood itself (Hinshaw and others 1977).

Neoplasia

Hypoglycaemia is a commonly reported complication of canine pancreatic neoplasia, but may also present in extrapancreatic neoplastic conditions including hepatocellular carcinoma, pulmonary carcinoma, haemangiosarcoma, plasmacytoma and melanoma (Kruth and Carter 1990). Hypoglycaemia has also been described in dogs with gastrointestinal leiomyoma or leiomyosarcoma (Bagley and others 1996).

1.4.3 Glucose perturbations in malaria

Malaria is an important disease worldwide and is the cause of 2.7 million human deaths every year (World Health Organisation, 1996). *Plasmodium falciparum* is the main aetiological agent in severe disease. Other causes of

malaria (*P. vivax*) cause serious illness, but rarely result in death. Falciparum malaria may manifest clinically in children as mild, moderate or severe disease, which may be uncomplicated or complicated. Patients with moderate disease require parenteral treatment, but are unlikely to develop severe disease once treated with appropriate antimalarial drugs. Patients with moderate disease have none of the defining features of severe disease, namely hypoglycaemia, lactic acidosis, coma, or seizures.

Severe illness leading to death is most likely in children between 1 and 4 years of age. By this age maternally derived protection has waned, and acquired immunity has not yet developed (Newton and Krishna 1998). Subsequently children develop immunity and severe disease in adults is unlikely in endemic situations. Severe disease may present in children as any of the following syndromes, alone or in combination: severe malarial anaemia, malaria with hyperpnoea, or cerebral malaria. Malaria may be further complicated by metabolic derangements such as hypoglycaemia and lactic acidosis (Newton and Krishna 1998). Hypoglycaemia has been recognised as a common and serious complication in severely ill human patients with malaria since as early as 1944 (Fitz-Hugh and others 1944). Children (Agbenyega and others 2000; Dzeing-Ella and others 2005) and pregnant women (Davis and others 1994) are at greater risk of developing hypoglycaemic complications. Hypoglycaemia is present in 20% of children with cerebral malaria (Newton and Krishna 1998), and was found in five out of ten pregnant woman with cerebral malaria in a watershed study conducted in Thailand in 1983 (White and others 1983). Hypoglycaemia has also been

found to be a common feature of murine models of malaria (Elased and Playfair 1994). Severe hypoglycaemia in mice infected with *Plasmodium chabaudi* and *P. yoelii* was associated with concurrent hyperinsulinaemia. Lactic acidosis complicates 35% of severe childhood malaria (Krishna and others 1994). This metabolic derangement commonly coexists with hypoglycaemia, and each independently defines severe disease and predicts fatality in children, adults (Agbenyega and others 2000) and pregnant woman (Phillips 1989; Manish and others 2003).

The similarities between canine babesiosis and human malaria are striking (Maegraith and others 1957; Welzl and others 2001). These diseases share numerous clinical and pathophysiological characteristics, as do human babesiosis and malaria (Clark and Jacobson 1998; Reyers and others 1998). Haemolysis, severe systemic inflammation and associated pro-inflammatory cytokine production are thought to contribute to disease pathogenesis in both babesiosis and malaria (Reyers and others 1998).

Pathophysiology of hypoglycaemia in malaria

The pathogenesis of malarial hypoglycaemia is complex and may be multifactorial. No single mechanism has been consistently implicated. Both malaria itself and antimalarial drug therapy (quinine) contribute to this metabolic derangement (Agbenyega and others 2000). Proposed contributing factors include hyperinsulinaemia (often, but not exclusively, following

treatment with quinine), increased anaerobic glycolysis, parasite metabolic demands for glucose, decreased hepatic blood flow and compromised hepatic function, endotoxin, inhibition of gluconeogenesis and 'malaria toxin'. The following pathophysiologic mechanisms have been suggested:

Increased anaerobic glycolysis

The presence of cellular hypoxia in severe systemic diseases such as malaria has been proposed to explain the hypoglycaemia and hyperlactataemia seen here. The observed sequestration of parasitised erythrocytes in small vessels with resulting capillary obstruction and hypoperfusion of tissues has been suggested as a cause for this hypoxia. Tissues are forced to rely on anaerobic glycolysis for the production of cellular ATP, significantly raising glucose consumption and lactate production in these tissues (Krishna and others 1994). Hypoglycaemia associated with higher white cell count has been seen in children with malaria (Taylor and others 1990).

Parasite demands for glucose

Glucose consumption (and the resulting lactate production) by intra-erythrocytic *P. falciparum* parasites has been proposed as a significant cause of hypoglycaemia in people with malaria (Krishna and others 1994).

Decreased hepatic blood flow

Sepsis and experimental infusion of TNF are associated with a dramatic reduction in hepatic blood flow. Considering that the liver is a large organ that

demonstrates a net uptake of lactate, decreased hepatic perfusion could be an important factor in the development of raised serum lactate (Clark and others 1997). It is conceivable that decreased hepatic perfusion could result in decreased glucose synthesis and hypoglycaemia.

Inhibition of gluconeogenesis

Since blood glucose concentration is a product of glucose production and glucose utilisation, it has been suggested that impaired gluconeogenesis may play an important role in the development of hypoglycaemia. Cytokines have been shown to inhibit hepatic gluconeogenesis in the mouse and human being during infection with malaria (Clark and others 1997). However, recent work has discounted this theory. Indeed, a profound rise in gluconeogenesis has been documented in malaria patients (van Thien and others 2006).

1.4.4 Glucose perturbations in Babesiosis

Babesiosis in South Africa is a common and serious disease caused by the virulent tick-borne haemoprotozoan parasite *Babesia rossi*. Another subspecies, *Babesia vogeli,* has been described in South Africa (Matjila and others 2004), but the virulence and role of the parasite in clinical disease is uncertain.

The disease is routinely categorised as mild uncomplicated, severe uncomplicated and complicated according to the clinical presentation. Dogs

typically present with signs attributable to acute haemolysis. They include fever, depression, anorexia, pale mucous membranes, splenomegaly and a waterhammer pulse. In patients with life-threatening anaemia the packed cell volume (PCV) drops below 15% (severe uncomplicated babesiosis).

Well-recognised complications of severe babesiosis include acute renal failure (Lobetti and Jacobson 2001), cerebral babesiosis, coagulopathy including disseminated intravascular coagulation (DIC), hepatopathy and icterus, immune-mediated haemolytic anaemia, pulmonary oedema, and haemoconcentrating babesiosis ('red biliary') (Jacobson and Clark 1994). Acute pancreatitis (Mohr and others 2000), systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) have also been described (Welzl and others 2001). Metabolic derangements are common including mixed acid-base disturbances (Leisewitz and others 2001), and hyperlactataemia (Leisewitz and others 2001; Nel and others 2004; Jacobson and Lobetti 2005).

In a recent study, 15% (38/250) of dogs presenting with severe babesiosis were hyperglycaemic (Keller and others 2004). This was thought to reflect the hypermetabolic nature of babesiosis (see 1.4.1 above), and was not a surprising finding. Hyperglycaemia was not correlated with a poorer prognosis in that study. In the same study, hypoglycaemia was identified as a metabolic complication in 19.8% (22/111) of severely ill dogs with babesiosis. Its occurrence was correlated with severe anaemia, an age of less than six

months, vomiting and icterus. As discussed previously, this phenomenon is also a clinical presentation of malaria patients, especially children.

Pathophysiology of hypoglycaemia in babesiosis

As in malaria, many factors may play a role in the pathogenesis of hypoglycaemia in babesiosis (Clark and Jacobson 1998). It stands to reason that hyperinsulinism as a cause of hypoglycaemia in malaria may similarly be found to be present in dogs with babesiosis.

1.5 Hyperinsulinism

1.5.1 Insulinoma

Hyperinsulinaemia in veterinary patients most commonly is associated with the presence of a functional pancreatic β cell neoplasm, or insulinoma. Insulin is autonomously secreted directly from neoplastic β cells, bypassing inhibitory signals usually exerted on the release of insulin during hypoglycaemia. Insulin concentrations may be severely elevated, and clinical signs usually result from persistent hypoglycaemia (Feldman and Nelson 2004).

1.5.2 Hyperinsulinism in malaria

Quinine-induced hyperinsulinaemia

The antimalarial drug quinine has been found to induce the secretion of insulin in patients with severe malaria (up to 10% of severely ill patients given quinine are affected). This effect is amplified in pregnancy; 50% of woman treated with quinine for severe malaria develop profound hypoglycaemia (Looareesuwan and others 1985). This impairment of the counter-regulatory response to insulin-induced hypoglycaemia may be due to suppression of glucagon and norepinephrine secretion (Connolly and others 2004). Hyperinsulinaemia and hypoglycaemia unrelated to quinine treatment have been described (Looareesuwan and others 1985; Shalev and others 1992).

Do *P. falciparum* parasites produce insulin-like molecules?

Substances known as inositol phosphoglycans (IPGs) have been shown to possess insulin mimetic properties. IPGs mimic several insulin actions and may constitute an insulin second messenger system (Caro and others 1996; Elased and others 2001). These IPGs were found in extracts of *Plasmodium falciparum* and *P. chabaudi.* In addition to being insulin mimetic, these substances may also promote the release of insulin (Elased and others 1996; Elased and others 2001). In these experiments, Elased *et al* reversed Type 2 diabetes in mice by injecting them with extracts from malaria-parasitized erythrocytes. This effect was not obtained by injecting non-parasitized red cells. Similarly, in a human Type 2 diabetic patient*,* P*. falciparum* infection

induced hypoglycaemia not related to quinine therapy (Shalev and others 1992). Taylor *et al* have also demonstrated the insulin mimetic properties of malaria toxin (Taylor and others 1992). These findings suggest a role for parasite-associated insulin mimetic substances (parasite-derived secretagogues) in the pathogenesis of hypoglycaemia in malaria. Further investigation is warranted.

Stimulation of insulin secretion by cytokines

As discussed earlier, hypoglycaemia in falciparum malaria has been associated with a poor prognosis and increased risk of mortality. Disease severity and death are also correlated with high circulating TNF concentrations. Although TNF may be produced in excessive amounts (see below), along with other cytokines including interferon-γ, lymphotoxin (LT), and IL-1, TNF itself does not appear to be responsible for the hyperinsulinaemia seen in rodent malaria (Elased and others 1996). Similar results were obtained in people (Manish and others 2003). Other cytokines may however play a role. IL-1 and IL-6 may act synergistically to produce hyperinsulinaemia and hypoglycaemia (Elased and others 1996). Further studies are required.

Malaria toxin and the 'cytokine theory' of malarial pathogenesis

The idea that the excessive toxin-induced production and secretion of pro-inflammatory cytokines (such as TNF, IL-1, LT and INF-γ) as being at the heart of the pathophysiology of many of the manifestations of malaria has been proposed and developed by Clark *et al*, and has been reviewed (Clark and others 1997). The theory has become known as the 'cytokine theory' of malaria. The theory provides arguments for the role of cytokines and nitric oxide in the development of coma in cerebral malaria, as well as malarial tolerance. The origins of the hypoglycaemia and hyperlactataemia described in malaria are also discussed in terms of this theory. This has relevance to carbohydrate metabolism in malaria:

TNF causes increased uptake (by promoting expression of GLUT1 transmembrane glucose transporters) and utilization of glucose (Clark and others 1997). Cytokines also stimulate the production of fructose 2,6-bisphosphate which activates phosphofructokinase, the major rate-controlling enzyme in glycolysis (Clark and others 1997). TNF has been shown to induce a futile substrate cycling of fructose 6-phosphate and fructose 1, 6-bisphosphate, with an attendant increase in the rate of glycolysis (Zentella and others 1993).

1.5.3 Hyperinsulinism in babesiosis

Causes for hyperinsulinaemia in canine babesiosis may include any of those suggested to play a role in human malaria. Pancreatitis has been identified in dogs with babesiosis, and the inflamed pancreas is potentially a source of excess insulin.

The inflamed pancreas as a source of insulin

Eighteen of 76 dogs (23%) with babesiosis had serum biochemical evidence of acute pancreatitis (Mohr and others 2000). These animals were not evaluated for hypoglycaemia. Pancreatic inflammation is however an unlikely cause of hyperinsulinism as people (Mizushima and others 2004) and rats (Abe and others 2002) with acute pancreatitis are usually glucose intolerant and require insulin therapy. Hyperglycaemia has been associated with a poor prognosis in dogs with spontaneous acute pancreatitis (Ruaux and Atwell 1998). Hypoglycaemia was present in 39.1% of dogs with acute pancreatitis (Hess and others 1998), which may have resulted from insulin treatment, sepsis, concurrent liver disease, or breed-related differences in metabolism.

Other causes for hypoglycaemia in babesiosis

Numerous pathophysiological mechanisms (in addition to hyperinsulinism) have been proposed to explain the hypoglycaemia seen in this disease. It is likely that the cause of hypoglycaemia in both malaria and babesiosis is multifactorial. Many individual factors and pathophysiological mechanisms

such as prolonged anorexia with depletion of hepatic glycogen reserves, young age, parasite metabolic demands for glucose, host hypermetabolism and white cell respiratory burst, relative adrenal insufficiency, and other factors are likely to contribute to the development of hypoglycaemia in babesiosis.

1.5.4 Treatment implications of hyperinsulinaemia in babesiosis

It has been shown that treating septic patients with a combination of glucose, insulin, and potassium reduces morbidity and mortality. A finding of hyperinsulinaemia and hypoglycaemia in dogs with severe babesiosis might suggest that a similar approach might be adopted in treating this disease. Furthermore, the administration of glucagon to hypoglycaemic patients may prove beneficial. If there is a significant subset of hypoglycaemic and concurrently hyperinsulinaemic dogs with severe babesiosis, therapy in these animals should be aimed at lowering insulin rather than administration of a glucose infusion alone which may serve to further stimulate insulin secretion (Elased and Playfair 1994).

Understanding the nature of the relationship between glucose and insulin in severe babesiosis is thus an important step in elucidating the pathophysiology underlying the development of hypoglycaemia frequently encountered as a life-threatening complication of this widespread endemic disease of dogs in South Africa.

CHAPTER 2: OBJECTIVES

This study seeks to investigate the role of insulin in the pathogenesis of the blood glucose abnormalities seen in dogs suffering from virulent babesiosis.

2.1 Problem Statement

Blood insulin concentration and its relationship (whether appropriate or inappropriate) to blood glucose concentrations are unknown in canine babesiosis.

2.2 Research Question

Are there differences in blood insulin concentrations between normoglycaemic, hypoglycaemic and hyperglycaemic dogs suffering from virulent babesiosis?

2.3 Hypothesis

Hypoglycaemia is associated with hyperinsulinism in severe canine babesiosis.

2.4 Benefits of this Study

The existence of an aberrant relationship between blood glucose concentrations and blood insulin concentrations has direct therapeutic implications in the clinical management of severe cases of canine babesiosis. Addressing the underlying hyperinsulinaemia, if present, rather than simply providing therapeutic glucose, may allow more effective management of life-threatening hypoglycaemia. Hyperglycaemia, on the other hand, may also be detrimental, and its identification and treatment with insulin may improve outcome.

Furthermore, by confirming or discounting the hypothesised relationship between glucose and insulin in cases of severe babesiosis, this study will serve to guide future research into the pathophysiology of deranged carbohydrate metabolism in babesiosis.

This study serves as partial fulfilment of the requirements for the principal investigator's MMedVet(Med) degree.

3: MATERIALS AND METHODS

3.1 Study Population

This study was a prospective, cross-sectional, observational study involving clinical cases. Dogs presented to the Outpatients clinic of the OVAH and diagnosed clinically with naturally acquired babesiosis were included in the study population. The study was reviewed and approved by the institutional Animal Use and Care Committee (protocol number V070/05).

3.1.1 Inclusion criteria

This study included dogs of any age, weight, breed or sex with clinical signs consistent with clinical babesiosis and *Babesia sp* parasites evident on peripheral blood smear.

3.1.2 Exclusion criteria

The following were considered criteria for exclusion of cases from the study. Dogs having received treatment with drugs affecting blood glucose concentration, e.g. intravenous glucose-containing fluids (dextrose), corticosteroids, other glucose-containing preparations (oral rehydration preparations, glucose powder) in the week prior to presentation; treatment with calcium-containing preparations as calcium may affect insulin release from the pancreas; administration of catecholamines or other adrenergic drugs; dogs receiving insulin therapy i.e. diabetic patients; dogs with pre-

existing chronic hepatic disease; dogs with hyperadrenocorticism (Cushing's syndrome) or hypoadrenocorticism (Addison's disease); co-infection with *Ehrlichia canis* as identified by polymerase chain reaction (PCR).

3.2 Clinical examination and neurological status

The individual ages and bodyweights of dogs were recorded at presentation and the owners completed a questionnaire documenting the duration of illness and time since last meal (Addendum A). The owner was issued with a Client Information Sheet (Addendum B), and informed consent was obtained (Addendum C). Clinical suspicion of infection with *B. rossi* was confirmed by identification of parasitised red blood cells on thin peripheral blood smears stained with Kyro-Quick™ stain[a]. The author verified the presence of parasites on blood smears. The following historical information was collected from the owner: signalment, prior medical history, duration of illness, and time since last meal. Clinical data, including rectal temperature, pulse rate, respiratory rate, clinical examination parameters including chest auscultation and abdominal palpation, presence of abdominal pain, vomiting, icterus, PCV, total

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[a] Kyron Laboratories, Benmore, South Africa

serum protein (TSP) and in-saline agglutination status, were collected at the time of presentation (Addendum D).

The neurological status of the patient was clinically assessed and dogs were recorded as having a normal habitus, or being depressed, weak, collapsed, comatose, or having seizures.

3.3 Sampling

Pre-treatment central venous blood samples were collected at the time of presentation by routine jugular venipuncture using pre-cooled syringes and transferred immediately into cooled 4 mL ethylenediaminetetraacetic acid (EDTA) and sodium fluoride-anticoagulated plastic tubes (Vacutainer™, BD Vacutainer Systems, Plymouth, UK). Samples were kept on ice until processing. As a routine clinical intervention, a drop of blood was screened for blood glucose concentration at presentation using a handheld glucometer[a]. Samples were centrifuged at 4°C for 10 minutes within 1 hour of collection, and plasma was separated and initially stored at -18°C. Fluoride-anticoagulated samples were submitted for glucose determination. EDTA-anticoagulated plasma samples were transferred to a -80°C freezer and batched for subsequent insulin determination.

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[a] Ascentia Elite Diabetes Care System, Bayer (PTY) Ltd, Isando, South Africa

3.4 PCR and RLB

Confirmation of the parasite subtype as *B. rossi* (and not *B. vogeli*) was by PCR. All samples were screened for *E. canis* and *B. vogeli* using PCR and reverse line blot (RLB) as previously described (Matjila and others 2004). PCR was conducted with a set of primers that amplified a 460-540 base pair fragment of the 18S small subunit (SSU) ribosomal RNA (rRNA) spanning the V4 region conserved for *Babesia* and *Theileria*. The *Ehrlichia* PCR amplified the V1 hypervariable region of the 16S SSU rRNA (Schouls and others 1999; Bekker and others 2002). The membrane used for RLB included probes for *B. vogeli, B. rossi, B. canis* and *E. canis*.

3.5 Groups

Animals were grouped into one of three groups according to their plasma glucose concentration (Table 3). Normoglycaemia was defined as blood glucose concentrations in the range 3.3-5.5 mmol/L. Glucose values in the hypoglycaemic group included values below 3.3 mmol/L (Turnwald and Troy 1983; Walters and Drobatz 1992), and hyperglycaemia was defined as blood glucose concentration >5.5 mmol/L (Keller and others 2004). Severe hypoglycaemia was considered to include values below 2.2 mmol/L. Severe

hyperglycaemia (above renal threshold) included glucose concentrations greater than 12 mmol/L.

Group	Glucose Concentration
Hypoglycaemic	<3.3 mmol/L
Normoglycaemic	3.3-5.5 mmol/L
Hyperglycaemic	>5.5 mmol/L

Table 3. Plasma glucose cut-off values in the three glucose groups

3.6 Glucose assay

Fluoride-anticoagulated samples were submitted to the Clinical Pathology laboratory of the Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, Onderstepoort, for glucose determination. Samples which could not be processed immediately were centrifuged and the plasma stored at -18°C. Storage time of the separated plasma ranged from 0 to 3 days. Glucose determination was carried out using a NExCT™ Clinical Chemistry System[a] via the hexokinase method (Sonnenwirth and Jarett 1980; Kaplan and Pesce 1984).

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[a] Alfa Wassermann B.V., Woerden, Netherlands

3.7 Insulin assay

EDTA- anticoagulated plasma samples were transferred to a -80°C freezer and batched for subsequent insulin determination. Plasma insulin assays were performed at the Hormone Laboratory, Section of Reproduction, Faculty of Veterinary Science, Onderstepoort, using a commercially available solid-phase radioimmunoassay (RIA) kit (Coat-A-Count®, DPC, Los Angeles, CA) previously validated for use in dogs (Kaplan and Pesce 1984; Parsons and others 2002).

The normal range for canine plasma insulin concentration is 35-180 pmol/L (Reimers and others 1982; Parsons and others 2002). Values below 10.7 pmol/L were considered to be below the limits of detection of the assay (Parsons and others 2002), and were entered in the statistical analysis as 10.7 pmol/L. Animals with a plasma insulin concentration above 180 pmol/L were considered to be hyperinsulinaemic.

Assay validation

The biological specificity of the insulin RIA was determined using three healthy greyhounds. The dogs were starved for 24 hours prior to obtaining a basal plasma sample, using the technique described under section 3.3 above.

An intravenous 50% dextrose solution was then administered to the dogs at a dosage of 1mL/kg, and a second plasma sample was obtained forty five minutes later. The basal and post-dextrose plasma samples were assayed for glucose and insulin concentrations. In addition, two plasma samples from a dog with persistent hypoglycaemia due to histologically confirmed, untreated functional pancreatic β cell neoplasia were also assayed for insulin concentrations.

Addition of an insulin degrading enzyme (IDE) inhibitor

Of the 98 plasma samples assayed for insulin concentration, twenty were aliquoted into duplicate samples, to which 10 µL of a 0.03 M solution of the insulin degrading enzyme (IDE) inhibitor *n*-ethylmaleimide (NEM) was added (final NEM concentration in plasma $1x10^{-3}$ M) immediately following centrifugation and separation of plasma samples. These samples were assayed for insulin in parallel with samples containing no NEM.

3.8 Data analysis

Results of insulin and glucose determinations, along with clinical and historical data, were recorded for each case on a spreadsheet using Microsoft Excel®[b].

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[b] Microsoft Corporation

Statistical analysis – Parameters were tested for normal distribution using the one-sample Kolmogorov Smirnov test. Differences in the median values of the variables in the three glucose groups were analyzed for non-parametric data with the Kruskal Wallis test and subsequently using the Mann-Whitney U test for pair wise comparisons. Normally distributed data were analysed using one way ANOVA, with the Bonferroni correction for multiple comparisons. For all comparisons, differences were considered significant when $p < 0.05$. Values for non-parametric data in the text are given as median and interquartile range (IQR) and for parametric data as mean ± standard deviation (SD). For comparison of mean values, a two-tailed Student's t-test was used. Statistical analysis was performed using a commercial software package (SPSS 14.0, 2005, SPSS Inc, 233 S. Wacker Dr, Chicago, Illinois, 60606)

CHAPTER 4: RESULTS

A complete data set is provided in Addendum E.

4.1 Description of Study Population

4.1.1 Total number and reasons for exclusions

Over a period of approximately three months, 98 dogs were sampled as they presented successively to the OVAH. All dogs included in the study had large babesia parasites present on their blood smears. PCR produced three positive results for *B. vogeli,* resulting in the exclusion of these cases from the study. The results of one dog were also censored due to prior intravenous dextrose administration. Thus the data from 94 dogs were included in the statistical analysis.

4.1.2 Clinical data and neurological status

Median age for all dogs was 16 months (IQR 9-39 months). Body temperature was the only normally distributed variable and the mean body temperature was 39.1°C (range 32.7 – 41.1°C). The median pulse rate was 130 beats per minute (IQR 112 – 150). The median respiratory rate was 46 breaths per minute (IQR 33 – 60). The median number of days ill prior to presentation was 2 days (IQR 2-4 days), and the median time since last meal was 24 hours

(IQR 12-48 hours, data available for 87 dogs). Seventeen dogs (18%) were icteric, and 14 dogs (15%) demonstrated neurological signs, including collapse (11/14) and coma (3/14).

Median duration of illness, time since last meal, pulse and respiration rate did not differ between the groups. Hypoglycaemic dogs (median 13.5 months) were significantly younger than normoglycaemic dogs (median 24 months) (p = 0.04). Hyperglycaemic dogs (median 9 months) were also significantly younger than normoglycaemic dogs (p < 0.001). Both hypoglycaemic dogs (median 5.6 kg) and hyperglycaemic dogs (median 9kg) had significantly lower bodyweight than normoglycaemic dogs (median 20 kg) (p < 0.01 for both). Hypoglycaemic dogs (mean 37.7° C; range 33 – 40.2) had significantly lower body temperature than normoglycaemic dogs (mean 39.6°C; range 33 – 41.1) (p < 0.001), but did not differ significantly from hyperglycaemic dogs (38.8; range 32.7 – 40.4) (p = 0.077)

4.2 PCR and RLB

Ninety five of the 98 dogs sampled were positive for *B. rossi* on PCR. Infection with *B. vogeli* was identified in 3 of the 98 dogs (3%) sampled. These dogs were censored from the study. All displayed clinically mild disease (Table 4). No positive results were obtained for *E. canis.*

Case	Age (months)	Glucose (mmol/L)	Insulin (pmol/L)
B59	12	4.4	<10.7
B77	3.2	6.5	14.1
B95*	96	5.1	<10.7

Table 4. Plasma glucose and insulin concentrations of dogs infected with *B. vogeli* (*PCR positive for *B. rossi* and *B. vogeli*)

4.3 Plasma glucose concentrations

For the 94 dogs included in the statistical analysis, the range of the plasma glucose concentrations was 1.0-6.6 mmol/L, with a mean of 4.4 ± 1.26 mmol/L (mean ± SD), and a median value of 4.7 mmol/L (IQR 4.0-5.1 mmol/L). Animals were retrospectively assigned to one of three groups according to their plasma glucose concentration from samples collected at presentation as follows: hypoglycaemic ([glucose] < 3.3 mmol/L; n=16 [17%]), normoglycaemic ([glucose] 3.3-5.5 mmol/L; n=62 [66%]), and hyperglycaemic ([glucose] > 5.5 mmol/L; n=16 [17%]) (Table 5).

Group	n
Hypoglycaemic (<3.3 mmol/L)	16
Normoglycaemic (3.3-5.5 mmol/L)	62
Hyperglycaemic (>5.5 mmol/L)	16

Table 5. Number of dogs in the three plasma glucose groups.

4.4 Plasma insulin concentrations

Assay validation

Insulin concentrations in three healthy greyhound dogs were below the detection limit of the assay (<10.7 pmol/L) following a 24 hour fast (Table 6). Following intravenous dextrose administration, plasma insulin concentrations were elevated (Table 6). All three dogs were hyperglycaemic, while dogs 2 and 3 were hyperglycaemic and hyperinsulinaemic. A similar pattern was noted in the dog sampled during the study which had been treated with dextrose prior to blood collection (blood glucose concentration at presentation was 1.1 mmol/L as determined by a handheld glucometer), which had a

plasma insulin concentration of 220.2 pmol/L following intravenous dextrose administration.

	Glucose (mmol/L)	Insulin (pmol/L)
Dog 1- Basal	4.1	<10.7
Dog 1- Post dextrose	11.3	168.7
Dog 2- Basal	4.1	<10.7
Dog 2- Post dextrose	>25	373.4
Dog 3- Basal	4.3	<10.7
Dog 3- Post dextrose	22.0	353.2

Table 6. Plasma glucose and insulin concentrations in three healthy dogs after a 24 hour fast and following intravenous 5 % dextrose administration

Samples from a dog with β cell neoplasia contained elevated insulin concentrations consistent with reported values in canine patients with this condition (Feldman and Nelson 2004) (Table 7).

Sample number	Insulin (pmol/L)	Glucose (mmol/L)
1	243	1.8
2	645	1.4

Table 7. Plasma insulin and glucose concentrations from a dog with pancreatic β cell neoplasia.

Effect of addition of an IDE inhibitor

The IDE inhibitor NEM was added to duplicate plasma samples from 20 dogs enrolled in the study. The samples were assayed in parallel and the results shown in Table 8. No significant difference was found between the insulin concentrations of the samples with no inhibitor (mean ± SD 19.05 ± 15.96) and those with inhibitor (mean ± SD 15.21 ± 11.53), using a two-tailed Student's t-test (p= 0.389).

Case number	Plasma insulin concentration (pmol/L)	
	No inhibitor	**With inhibitor**
75	16.30	10.7
77	14.14	10.7
78	10.7	10.7
79	10.7	10.7
80	29.80	18.81
81	17.88	10.7
82	10.7	10.7
83	45.23	28.0
84	10.7	10.7
85	71.94	57.44
86	38.41	30.37

87	10.7	10.7
88	10.7	10.7
91	10.7	10.7
94	10.7	10.7
95	10.7	10.7
96	19.03	10.7
97	10.7	10.7
98	10.7	10.7
99	10.7	10.7

Table 8: Plasma insulin concentrations from 20 dogs with and without the addition of NEM (values below 10.7 pmol/L are below the detection limit of the assay)

Plasma insulin concentrations in dogs with babesiosis

The median plasma insulin concentrations (IQR in parentheses) for the hypoglycaemic, normoglycaemic and hyperglycaemic groups were 10.7 pmol/L (10.7-18.8 pmol/L), 10.7 pmol/L (10.7-29.53 pmol/L), and 21.7 pmol/L (10.7-45.74 pmol/L) respectively (Figure 1). The median insulin concentrations for the hypoglycaemic and normoglycaemic groups were below the detection limit of the assay. Although there was a trend for insulin concentration to increase as blood glucose concentration increased, no significant difference in

insulin concentration was found between the three groups (Chi-square$_{k\text{-}w}$ = 1.972, p = 0.373). Two dogs, one with concurrent hypoglycaemia, were found to have insulin concentrations above the reference range. One dog was mildly hyperinsulinaemic (case number 56; insulin concentration 198 pmol/L); the other was severely hyperinsulinaemic (case number 34; insulin concentration 1653 pmol/L).
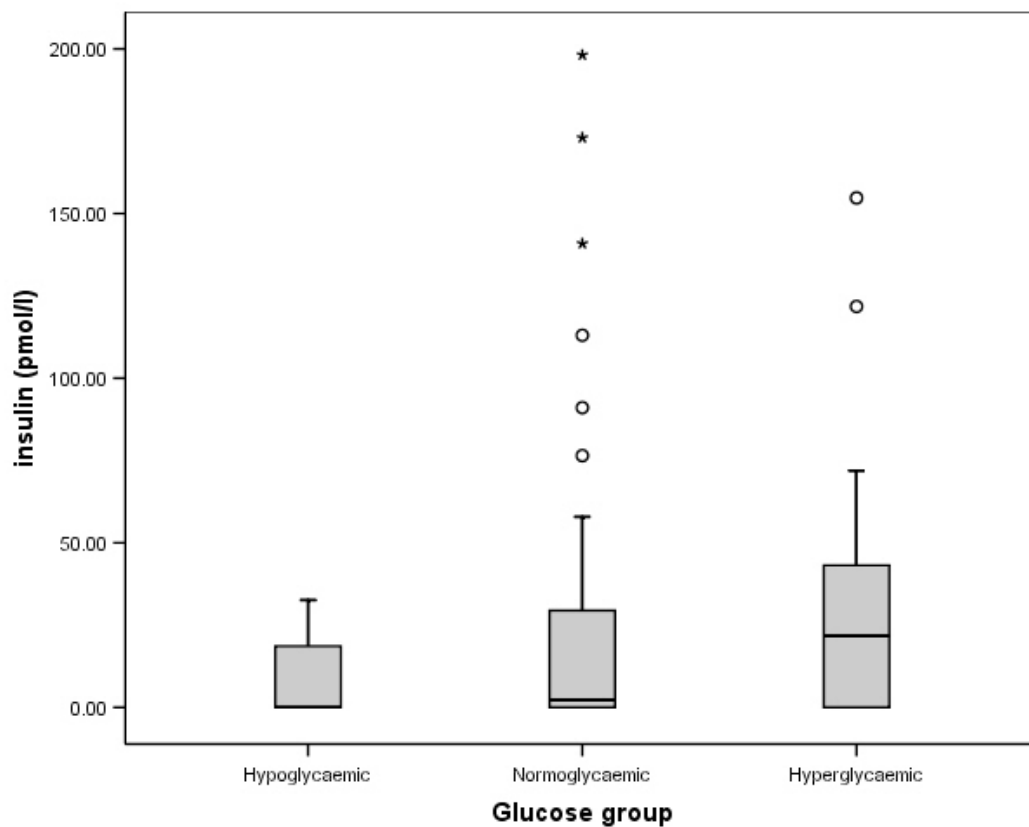
Figure 1. Boxplot showing plasma insulin concentrations for the three plasma glucose concentration groups. The box represents the interquartile range, the median is shown as a horizontal bar, and the T bars represent the main body of data. Outliers are indicated as open circles and stars, and the severely

hyperinsulinaemic case (insulin concentration 1653 pmol/L) is not shown here in order to allow a meaningful scale to be used in the figure.

Of the sixteen hypoglycaemic dogs, 6 had detectable plasma insulin concentrations (Table 9). Excluding case number 34, the range of insulin concentrations in the remaining 5 hypoglycaemic dogs was 18.45-32.6 pmol/L.

Case Number	Glucose concentration (mmol/L)	Insulin concentration (pmol/L)
5	2.4	10.7
18	2.2	18.45
26	3.0	10.7
31	1.3	10.7
34	2.9	1653
37	2.5	10.7
44	1.4	10.7
46	2.1	10.7
55	2.4	18.81
57	3.2	18.74
61	1.1	28.29
62	2.2	32.6

79	2.0	10.7
84	2.8	10.7
88	1.6	10.7
90	1.0	10.7

Table 9. Plasma glucose and insulin concentrations for 16 hypoglycaemic dogs with babesiosis. Values recorded as 10.7 refer to insulin concentrations below the detection limit of the assay.

CHAPTER 5: DISCUSSION

5.1 Babesia sp. parasites

Babesia rossi is a large babesia parasite which causes virulent canine babesiosis in South Africa, resulting in severe disease in susceptible individuals. Disease resulting from infection with this species was the focus of the current study, and it was therefore necessary to eliminate individuals from the study with disease resulting from other babesiae. South Africa is considered free of *Babesia gibsoni*. This small babesia has been detected here exclusively in animals imported from endemic areas (Matjila and others 2004). Only large babesia parasites were identified on blood smears from dogs included in this study. Indeed, no small babesia parasites have been detected in this country in many decades. It is therefore considered unlikely that *B. gibsoni* was present in any of the dogs in this study, and a probe for this small babesia parasite was not included in the RLB.

Babesia vogeli has recently been identified in South Africa with a local prevalence of approximately 4% in dogs screened using PCR (Matjila and others 2004). In countries where it is regularly isolated, *B. vogeli* usually causes mild or subclinical disease in adult dogs, with a more severe clinical entity affecting dogs younger than 10 weeks (Irwin and Hutchinson 1991). This study found a similar prevalence of 3% in the dogs sampled, and all dogs infected with *B. vogeli* had mild clinical disease.

5.2 Ehrlichia canis

The reported prevalence of *E. canis* in dogs in South Africa varies from 3% (relying on PCR detection) to 68% (serology). Prevalence of this bacterial disease is likely affected by geographical location and the dog's environment, and variation between rural and urban areas has been demonstrated (Eckersley and others 1992). Despite a high prevalence in the study area, *E. canis* was not detected in any of the samples submitted for PCR.

5.3 Glucose perturbations

Hypoglycaemia

Hypoglycaemia was present in 16 of 94 (17%) dogs included in this study. A proportion of these dogs showed signs consistent with neuroglycopoenia, including collapse and coma. Five of the 16 dogs with hypoglycaemia were icteric, a finding similar to those of a previous study (Keller and others 2004), where icterus was established as a significant risk factor for the development of hypoglycaemia. Hypoglycaemic dogs were significantly younger, with lower body weight (probably a function of younger age) than normoglycaemic dogs. In a previous study, dogs less than six months of age were 2.8 times more likely to develop hypoglycaemia (Keller and others 2004). The majority

(95.7%) of hypoglycaemic dogs in that study were hospitalised due to the presence of severe disease. Hypoglycaemic dogs in this study had significantly lower body temperatures than normoglycaemic dogs. Hypothermia is a feature of severe babesiosis (Jacobson and Lobetti 2005), and is likely due to the presence of circulatory shock (Jacobson and others 2000; Böhm and others 2006) and systemic inflammation in severely affected dogs with impending organ dysfunction (Reyers and others 1998; Welzl and others 2001).

In humans, children commonly present with hypoglycaemia in conjunction with a variety of severe diseases (Kawo and others 1990; Solomon and others 1994; Osier and others 2003), despite having fully developed gluconeogenic capacity at birth (Bier and others 1977), suggesting that glycogen stores may be limited. This may apply to young dogs, where lower concentrations of stored glycogen may reduce the host's ability to withstand the physiologic stresses of severe disease, and such animals cannot adequately compensate during periods of increased demand for carbohydrate fuels.

In human patients with trauma and sepsis, a significant increase in glucose consumption is mediated by release of inflammatory cytokines such as TNF from the macrophage-rich spleen, liver and lungs (Mizock 1995). In human malaria, a disease pathophysiologically similar to canine babesiosis, increased anaerobic glycolysis due to microvascular obstruction by sequestrated parasitised red blood cells, parasite demands for glucose, decreased hepatic blood flow, compromised hepatic function, and failure of

gluconeogenesis may contribute to the pathogenesis of hypoglycaemia (White 2003; Jacobson and Lobetti 2005). Similar mechanisms are likely to contribute to the development of hypoglycaemia in dogs with severe canine babesiosis.

Due to the relatively small size of the hypoglycaemic group (n=16), the incidence of insulin-induced hypoglycaemia in canine babesiosis may have been underestimated by this study. Further sampling of greater numbers of hypoglycaemic dogs may identify more dogs with hyperinsulinaemia. This study was cross-sectional, and did not follow temporal patterns in blood glucose and insulin concentrations. A more detailed understanding of fluctuations in the glucose: insulin relationship during the natural history of this disease might be gained if these parameters were to be measured in dogs not only at presentation, but also at various points following admission. However, the results of such a clinical study would be markedly confounded by the subsequent administration of varying doses of dextrose and other drugs. In addition, glucocorticoids are used to treat the secondary immune-mediated haemolysis present in some cases. Therefore, such a study would only be feasible if conducted in experimentally infected dogs that remain untreated, with its attendant ethical considerations.

Hyperglycaemia

Up to 71% of human beings suffering from critical illness will be hyperglycaemic (Capes and others 2000). Blood glucose concentrations ranging between 6.7 and 11.2 mmol/L have been suggested as a definition of

critical illness-associated stress hyperglycaemia in people (Mizock 2001). Using a similar cut-off (greater than 6.6 mmol/L), stress hyperglycaemia was identified in 38 (16%) of 245 critically ill dogs (Torre and others 2007). Nine (38%) of these dogs had blood glucose concentrations between 8.8 and 11.0 mmol/L, and 4 (11%) had blood glucose concentrations higher than 11 mmol/L. Although hyperglycaemia (glucose concentration >5.5 mmol/L) occurred in 16 (17%) of the dogs in the present study, the degree of hyperglycaemia was consistently mild, the highest glucose concentration being only 6.6 mmol/L. Thus, using the definition employed by Torre *et al*, no dogs could be considered to have true illness-related stress hyperglycaemia. A similar pattern was observed in another study (Jacobson and Lobetti 2005), where the glucose concentrations in 15 non-hypoglycaemic dogs was 5.1± 1.0 mmol/L (mean ± SD). Keller *et al* reported hyperglycaemia in 38/250 (15%) of dogs at presentation (Keller and others 2004). Only 55% of the hyperglycaemic dogs were regarded to be ill enough to justify admission to the hospital. In all, hyperglycaemia was mild. Since virulent babesiosis causes severe systemic illness, the low observed prevalence of severe hyperglycaemia in canine babesiosis is surprising.

5.4 Plasma insulin concentrations

Insulin determination methods and the effects of haemolysis

Insulin determination methods in canine serum or plasma samples currently employ radioimmunoassay (RIA) techniques (Reimers and others 1982). Haemolysis is known to negatively affect insulin RIA results, usually dramatically lowering available immunoreactive insulin (IRI). This is as a result of an IDE or insulinase present within erythrocytes, which is released into plasma as a result of haemolysis. IDE cleaves IRI thereby rendering it unavailable for reaction in the RIA (Sapin and others 1998). This phenomenon presents a major problem, because canine babesiosis is a haemolytic disease. Plasma samples from dogs with babesiosis routinely have free haemoglobin concentrations of 2-6 g/L (M Nel-unpublished data). These concentrations are adequate to cause interference with the RIA test. Haemolysed samples containing 5g/L haemoglobin stored for 24 hours at 4ºC showed a decrease in insulin of 11% (Sapin and others 1998). In the same study the authors overcame this problem by adding the sulfhydryl-modifying reagent *p*-chloromercuriphenylsulfonic acid at a final concentration of 1 mmol/L to serum or plasma. The authors suggest handling samples on ice and centrifuging at 4ºC with prompt analysis or freezing to limit insulin degradation. Careful attention was paid to the handling, storage and processing of plasma samples obtained in the current study. Blood was drawn using cooled syringes and needles, and processed rapidly at 4°C before

freezing, as previously recommended (Sapin and others 1998). These procedures minimize insulin degradation in haemolysed samples.

No difference was found in the insulin concentrations of duplicate samples with or without added NEM, leading the author to conclude that, despite the frequent presence of haemolysis in our plasma samples, the sample handling techniques employed here resulted in minimal insulin degradation, allowing the accurate determination of insulin concentrations in plasma samples from dogs with babesiosis.

Insulin RIA validation

Insulin concentrations in three healthy greyhounds were low (below the detection limit of the assay) following a 24 hour fast. An appropriate rise in insulin concentration was documented following dextrose administration. All three dogs showed a supra-physiological insulin response to rapidly rising blood glucose concentrations following dextrose administration. Similarly, hyperinsulinaemia occurred in the one dog with babesiosis which was sampled after receiving intravenous dextrose, again indicating a strong pancreatic β cell response. The dose of dextrose administered to this patient is not known, but the hyperinsulinaemia is likely to have resulted from overzealous dextrose administration. Insulin concentrations were in the

expected range in plasma samples from a dog with a confirmed functional pancreatic β cell neoplasm.

Relationship between glucose and insulin concentrations

The results of this study confirm that, for the most part, insulin concentrations in dogs with babesiosis are low, which is similar to people with severe malaria (Looareesuwan and others 1985; White 2003; van Thien and others 2006). Low insulin concentrations can therefore be considered a normal finding in the ill, anorexic dogs studied here. In the dogs sampled here the median insulin concentration in the hypoglycaemic and normoglycaemic groups was below the detection limit of the radioimmunoassay, whereas the median insulin concentration in the hyperglycaemic group was below the lower limit of the reference range. An apparent trend for insulin concentration to increase as blood glucose concentration increased was identified. These results suggest that insulin secretion was inhibited in the hypoglycaemic and normoglycaemic dogs, and that an appropriate physiological relationship exists between glucose and insulin in this group of dogs with babesiosis.

Fasting plays a major role in the suppression of insulin secretion. In healthy dogs, fasting results in mild hypoglycaemia with moderate decreases in insulin concentration (de Bruijne and others 1981). It has recently been suggested that starvation may play a major role in the pathogenesis of hypoglycaemia in

malaria (van Thien and others 2006). Dogs suffering from babesiosis are usually anorexic as a result of their illness, and frequently have not eaten in 24-48 hrs. They are probably also severely glycogen-depleted. In conjunction with increased metabolic demands for glucose and decreased hepatic gluconeogenesis, fasting may contribute significantly towards the development of hypoglycaemia. Dogs suffering from babesiosis can thus be expected to have appropriately low plasma insulin concentrations. When blood glucose concentrations fall further (below 2.8 mmol/L), insulin secretion is completely inhibited (de Bruijne and others 1981; Leifer and Peterson 1984; Walters and Drobatz 1992; Feldman and Nelson 2004). Insulin should therefore be practically undetectable in the plasma of hypoglycaemic patients, due to a lack of direct β cell stimulation, and an increase in α-adrenergic inhibition of pancreatic insulin secretion (Feldman and Nelson 2004).

Six of the 16 hypoglycaemic dogs did, however, have detectable plasma insulin concentrations. For these dogs (excluding the severely hyperinsulinaemic dog, case number 34 discussed below), the range of insulin concentrations was 18.45-32.6 pmol/L. It has been suggested that a plasma insulin concentration greater than 72 pmol/L, with a concurrent glucose concentration less than 2.8 mmol/L, constitutes an inappropriate excess of insulin (Feldman and Nelson 2004). Since none of the hypoglycaemic dogs had plasma insulin concentrations greater than 72 pmol/L, none can be said to have had inappropriately high concentrations of insulin. On the contrary, the values obtained were below the reference range reported for dogs, i.e. 35-180 pmol/L (Reimers and others 1982; Parsons and others 2002). These findings

are similar to those in murine malaria models, where insulin secretion is appropriately suppressed (Holloway and others 1991).

Hyperinsulinaemia

Hyperinsulinaemia was identified in two cases, and was present in only one dog in conjunction with hypoglycaemia. The first of the hyperinsulinaemic dogs, a six-year-old intact male Fox Terrier (case 56; insulin concentration=198 pmol/L; glucose concentration= 4.8 mmol/L), had a plasma insulin concentration marginally above the reference range. The dog was suffering from complicated babesiosis, was comatose and severely icteric, and died shortly after presentation. Considering that the dog was comatose at presentation it is unlikely to have eaten in the hours immediately prior to blood sampling, and postprandial insulin release is therefore unlikely. Peripheral insulin resistance commonly coexists with hyperinsulinaemia in cases of human malaria (Davis and others 1990; van Thien and others 2006), and may account for the normal blood glucose concentration associated with hyperinsulinaemia in this dog.

In the second dog with hyperinsulinaemia, a 2-year-old male Boxer (case 34; insulin concentration= 1653 pmol/L [similar value obtained on repeat analysis]; glucose concentration= 2.9 mmol/L), the insulin concentration was dramatically higher than normal physiological values, and is higher even than

values commonly encountered in dogs with β cell neoplasia (Dunn and others 1992). This dog was also moderately hypoglycaemic, and was collapsed despite a haematocrit of 27%. This patient recovered and was discharged, and represents the only case identified in this study with concurrent hypoglycaemia and hyperinsulinaemia.

Besides insulin resistance, the most likely cause of hyperinsulinaemia in the two hyperinsulinaemic dogs described here would be an undiagnosed insulin-secreting pancreatic β cell neoplasm. No definitive tests were carried out to rule out this condition. However, the dogs were reported by their owners to be in good health prior to the babesiosis-related illness, with no prior history of signs suggesting insulinoma. In the case of the boxer (case 34), the dog was clinically normal for a period of twenty months following discharge, at which time it was euthanased for undiagnosed nasal disease. Another possible cause of hyperinsulinism is xylitol toxicity. Cross-reaction of insulin-like growth factors with the insulin assay may influence the radioimmunoassay. Measuring canine-specific C-peptide may have overcome this problem.

A small sub-population of dogs suffering from babesiosis may therefore exist in which inappropriate insulin secretion occurs, increasing the risk for hypoglycaemia.

CHAPTER 6: CONCLUSIONS

We conclude that hyperinsulinaemia is an unlikely cause of hypoglycaemia in dogs suffering from virulent babesiosis attributable to infection with *B. canis rossi*. A small sub-population of dogs may however exist in which significant hyperinsulinaemia could result in clinical hypoglycaemia. Future studies involving larger numbers of hypoglycaemic dogs and including assays for canine-specific C-peptide may assist in quantifying this phenomenon.

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ADDENDA

Addendum A

Client Questionnaire

Dear Sir/ Madam

In order to ensure the accuracy of data, please answer the following questions honestly and openly. If you have any questions or if anything is unclear, please do not hesitate to ask.

Details of study subjects   Date ____/____/____

Owner questionnaire   *dd   mm   yy*

Owner number: _____   Patient number: _____

IDENTIFICATION

1. Owner's name:

   _____

2. Owner's address:

   _____

                                        Number & Street

_____

_____

Province   Postal Code

3. Dog's name:

_____

4. Dog's birth date: ____/____/____   Dog's age:_____

                          dd   mm   yy

Please circle the appropriate answer:

1. Has your pet been diagnosed and/or treated for babesiosis (tick-bite
   fever/ 'bosluiskoors') in the last three weeks?………Yes   No
2. Has your pet received any other treatments or medications whatsoever
   in the last week?………………………………………..Yes   No
3. If you answered yes to the last question, please specify the treatments
   given…………………………………………………………….
4. What, how much and when did your pet last
   eat?…………………………………………………………………………….
5. Has your dog been vomiting during this current illness: Yes   No
6. Has your pet at any stage previously been diagnosed or treated for any
   of the following conditions:
Diabetes………………………………………………….Yes   No
Liver disease…………………………………………….Yes   No

Jaundice…………………………………………………….Yes   No

Hormonal conditions such as Cushing's syndrome…….Yes   No

7. If 'Yes' to any of the above questions, please give

   details…………………………………………………………

   …………………………………………………………………

   …………………………………………………………………

   …………………………………………………………………

Thank you for your time and cooperation.

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0110

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Addendum B

Client Information Sheet

Dear sir/ madam

Your dog has been diagnosed with babesiosis (biliary fever, bosluiskoors). This is caused by a small parasite (called *Babesia canis*) that lives inside the red blood cell. This disease is very similar to malaria that is seen in humans, however, babesiosis is transmitted by ticks and not by mosquitoes, as in the case of malaria.

As part of the fight against Babesia canis, the Department of Companion Animal Clinical Studies and Paraclinical Sciences are doing ongoing research on babesiosis. This will help dogs survive this terrible disease. In this research project, we are investigating the problem of high and low blood sugar often seen in dogs with babesiosis, and also the role of high or low insulin concentrations in the blood of these dogs. Insulin is the major hormone controlling blood sugar.

We would like your help by being allowed to include your pet in this project. Your dog will be treated the same as any other patient would be. The only difference is that we will take two blood samples for determining the glucose and insulin concentrations in your pets' blood. There will be no extra cost to you over and above the normal cost of treating your dog for babesiosis.

If you agree, you will be requested to fill in a questionnaire and give written consent to allow us to take blood from your dog. This procedure is safe and routine.

You may remove your pet from the study at any time. The Animal Use and Care Committee of the University of Pretoria has approved this study.

Thank you in advance

Dr. Phil Rees

Department of Companion Animal Clinical Studies

Faculty of Veterinary Science

Onderstepoort

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Addendum C

FORM FOR INFORMED CONSENT

I, _____, the undersigned owner / authorised representative (please delete), hereby give permission for the pet dog under my care:

Name:

Breed:

Age:

Sex:

Colour:

to participate in the study of measuring blood glucose and insulin concentrations.

I understand that blood will be collected from the above animal. I further understand that this is a routine and safe procedure. I also understand that the cost pertaining to this study is not my responsibility and that I am only liable for costs relating to the diagnosis, treatment and any complications or other cost that relate directly to the above animal suffering from babesiosis.

This study has been explained to me and I have been given the Information Sheet. I am further aware that I may remove my animal from this study at any time at my request and this will in no way jeopardise the proper care of my dog.

Signed at Onderstepoort on this _____ day of the month of _____ in the year

Name of owner or authorised representative   Signed

Name of witness   Signed

Addendum D

PHYSICAL EXAMINATION

- Temperature _____ ℃

- Pulse _____ per minute

- Respiratory frequency _____ per minute

FURTHER COMMENTS REGARDING PHYSICAL EXAMINATION

1. Habitus: (Please tick)

    Collapsed, unable to stand_____

    Weak, but able to stand_____

    Normal_____

2. Ht _____

3. Icterus (Please circle)

1+   2+   3+

4. Neurological signs

_____

5. Plasma Glucose concentration   Glucose group

_____

6. Lymphadenopathy

_____

7. Serum colour

_____

8. Urine colour

_____

Addendum E.

Complete data set for plasma glucose and insulin concentrations for 98 dogs with babesiosis. Values below 10.7 pmol/L were considered to be below the limits of detection of the assay, and are entered in the table as a value of 10.7 pmol/L.

CASE NO	INSULIN (pmol/L)	GLUCOSE (mmol/L)
B1	10.7	4.8
B2	10.7	5.5
B3	10.7	5.4
B4	10.7	5.8
B5	10.7	2.4
B6	10.7	6.4
B7	154.73	6.1
B8	29.44	5.0
B9	10.7	5.0
B10	10.7	4.7
B11	24.70	3.8
B12	10.7	4.7
B13	10.7	4.4
B14	31.95	6.6
B15	10.7	4.6

B16	27.50	6.2
B17	50.48	4.9
B18	18.45	2.2
B19	113.01	4.2
B20	10.7	4.9
B21	10.7	5.1
B22	37.98	5.7
B23	48.32	5.7
B24	28.07	5.3
B25	10.7	4.2
B26	10.7	3.0
B27	11.70	4.8
B28	43.51	5.0
B29	12.06	4.4
B30	10.7	3.4
B31	10.7	1.3
B32	57.87	4.0
B33	10.7	5.0
B34	1653.27	2.9
B35	173.04	4.9
B36	10.7	4.5
B37	10.7	2.5
B38	27.57	5.3
B40	10.7	4.4

B41	7.32	4.7
B42	10.7	3.8
B43	10.7	4.7
B44	10.7	1.4
B45	76.47	4.5
B46	10.7	2.1
B47	14.50	4.7
B48	32.60	5.6
B49	10.7	4.7
B50	57.80	5.0
B51	15.80	6.0
B52	10.7	5.1
B53	10.7	3.6
B54	140.87	4.0
B55	18.81	2.4
B56	198.17	4.8
B57	18.74	3.2
B58	55.93	4.5
B59	10.7	4.4
B60	10.7	5.7
B61	28.29	1.1
B62	32.60	2.2
B63	10.7	6.0
B64	17.52	5.0

B65	10.7	4.7
B66	10.7	4.2
B67	10.7	4.9
B68	36.40	4.7
B69	10.7	4.8
B70	10.7	4.5
B71	10.7	5.8
B72	121.77	5.7
B73	10.7	4.7
B74	10.7	4.7
B75	16.30	5.1
B76	91.04	4.2
B77	14.14	6.5
B78	10.7	5.7
B79	10.7	2.0
B80	29.80	4.7
B81	17.88	4.7
B82	10.7	4.2
B83	45.23	5.5
B84	10.7	2.8
B85	71.94	6.0
B86	38.41	3.7
B87	10.7	4.5
B88	10.7	1.6

B89	10.7	4.2
B90	10.7	1.0
B91	10.7	5.3
B92	10.7	5.0
B93	10.7	6.3
B94	10.7	3.6
B95	10.7	5.1
B96	19.03	4.9
B97	10.7	5.1
B98	10.7	5.1