Screening for prediabetes in senior cats and metabolomic characteristics of obesity and Burmese cats

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Abstract

Type 2 diabetes is the most common form of diabetes in humans and cats, and senior age and obesity are major risk factors. Diabetes is typically diagnosed in cats once clinical signs are evident. In humans, prediabetes is a metabolic state between normal glucose homeostasis and diabetes, and is diagnosed by demonstrating impaired glucose tolerance (IGT) and/or impaired fasting glucose (IFG). However, prediabetes has not yet been defined in cats, and reference values for these variables have not been reported for senior cats.

In this thesis, the first experiment investigated if the current practice of dosing glucose by bodyweight results in spurious effects on measures of glucose tolerance in obese cats. Glucose data from glucose tolerance and insulin sensitivity tests were analysed before and after 16 cats were fed ad libitum for 9 - 12 months to promote weight gain. Bodyweight and body condition score (BCS) were positively correlated with 2-min, 2-h glucose concentrations and glucose half-life following a glucose tolerance test (GTT) when glucose dosing was by bodyweight. To avoid obese cats being incorrectly classified as having impaired glucose tolerance when glucose was dosed on a bodyweight basis, equations were developed to adjust either glucose dose or 2-h glucose to compensate for obesity. This has important implications for clinical studies assessing the effects of interventions on glucose tolerance when lean and obese cats are compared.

The second experiment established methodology and cutpoints for fasting and 2-h glucose during a GTT in healthy, client-owned senior cats (≥ 8yrs, n=78), by using ear/paw samples and a portable glucose meter calibrated for feline blood. Fasting blood glucose was measured from ear/paw samples and glucose (0.5 g/kg bodyweight) administered IV, followed by blood glucose measurements at 2 min and 2 h. Cutpoints for fasting and 2-h glucose concentrations
were established as the upper limits of 95% reference intervals using cats with BCS 4 or 5 on a 9-point scale and were 6.5 mmol/L for fasting glucose and 9.8 mmol/L for 2-h glucose.

The third experiment established a methodology and reference interval for screening blood glucose in senior cats. Blood glucose was measured at the beginning of the consultation in clinically healthy cats (≥8 years, n=120) from an ear/paw sample using a portable glucose meter, and again after physical examination from a jugular sample. The cutpoint for screening blood glucose was 10.5 mmol/L. Mean screening blood glucose was greater than mean fasting glucose. Based on these results, it is recommended that cats with screening blood glucose from 6.5 mmol/L to 10.5 mmol/L be retested several hours later, and cats with screening values >10.5 mmol/L or a second test > 6.5 mmol/L should have fasting and 2-h blood glucose measured after overnight hospitalization.

Metabolomic markers of obesity and prediabetes have been investigated in humans, but there is limited data available in cats. Additionally, there is limited research on metabolomics in Burmese cats, which are reported to have dyslipidaemia and are 3-4 times more likely to develop diabetes than domestic cats. The last two experiments in the thesis address the effect of obesity and the Burmese breed on the metabolome.

A study was performed to identify metabolomic changes associated with obesity in senior cats, and to determine if such metabolites are associated with biochemical measures of abnormal glucose metabolism. Biochemical and GC-MS analyses were performed on blood samples from 21 lean and 18 obese non-Burmese cats. In obese cats, metabolomic analysis identified increased fatty acids (palmitoleic and myristoleic), glycerol, glycolic acid, and phenylalanine, whilst only the amino acid alanine was decreased. Palmitoleic acid was positively, and alanine negatively correlated (p<0.05) with leptin concentrations, and glycolic
acid and glycerol were positively associated (p<0.05) with insulin. This study is the first to report on metabolomic changes of obesity in senior cats.

The final study examined metabolomic differences between Burmese (n=19) and non-Burmese cats (n=30) by GC-MS analysis. In Burmese cats many glucogenic amino acids, together with hydroxyproline and aminobutyric acids were increased, whilst beta-alanine was decreased. Fructose and glucose, cholesterol and some fatty acids, glycerol-3-phosphate and various acids were all decreased in Burmese cats. Urea was also increased in Burmese. Burmese cats had increased insulin, triglyceride, and MCP-1 concentrations which had moderate positive associations with amino acids. Numerous relationships were identified between GC-MS metabolites and measures of glucose metabolism. This study is the first to report metabolite differences between healthy senior non-Burmese and Burmese cats.

The findings in this thesis have established the test methodology and reference intervals for fasting and screening blood glucose and glucose tolerance in senior cats. These results have direct application to clinical practice to facilitate earlier diagnosis of prediabetes in cats. Additionally, the identification of metabolite differences in cats associated with obesity or Burmese breed represents potential new biomarkers of metabolic dysfunction in cats at high risk of developing diabetes.
Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Contributions by others to the thesis

The work presented in this thesis is original and was carried out by the candidate except where acknowledged above, or as follows:

Professor Jacquie Rand (principal supervisor) provided intellectual input on the study design, interpretation of results and editorial comment of each of the chapters.

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I dedicate this thesis to my family.
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Glucose, prediabetes, glucose tolerance, obesity, screening, metabolomics, GC-MS, cats.

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List of abbreviations used in the thesis

ALT alanine aminotransferase
BCAA branched chain amino acids
BCS body condition score
CI confidence interval
CV coefficient of variation
DEXA dual-energy x-ray absorptiometry
DM dry matter
ELISA enzyme-linked immunosorbent assay
ESI-QqTOF-MS Electrospray ionisation quadrupole time of flight mass spectrometry
FFA free fatty acids
fPLi Feline pancreatic lipase
GC-MS gas chromatography- mass spectrometry
G:I ratio glucose: insulin ratio
GLP1 Glucagon like peptide-1
Gt serum glucose concentration at time t
GTT Glucose tolerance test
G0 initial serum glucose concentration based on extrapolation from the period from 15 to 90 min to time 0
h hours
HDL high density lipoprotein
HEC hyperinsulinaemic euglycaemic clamp
HMW high molecular weight (adiponectin)
HOMA homeostasis model assessment
HPLC High Performance Liquid Chromatography
IAPP Islet amyloid polypeptide
IGT impaired glucose tolerance
IFG impaired fasting glucose
IL-1 interleukin -1
IV intravenous
IVGTT intravenous glucose tolerance test
L:A ratio leptin to adiponectin ratio
LC-MS liquid chromatography- mass spectrometry
LDL low density lipoprotein
LMW low molecular weight (adiponectin)
MCP-1 Monocyte chemoattractant protein-1
ME metabolisable energy
min minutes
MinMod Minimal model technique
mo months
NATA National Association of Testing Authorities
NFE Nitrogen Free Extract
NMR Nuclear magnetic resonance
NMRI Naval Medical Research Institute
NOMIS Normalization using optimal selection of multiple internal standards
oGTT oral glucose tolerance test
OPLS orthogonal projections to latent structures
OSC Orthogonal signal correction
PCA Principal component analysis plots
PLS Partial least squares regression
PLS-DA Partial least squares discriminant analysis
RIA radioimmunoassay
RLA relative log abundance plot
SCD1 stearoyl-CoA desaturase-1
SD standard deviation
SEM standard error of the mean
SPME solid-phase microextraction
T ½ Half life
VLDL very low density lipoprotein
Chapter 1: Review of the Literature

Section 1: Diabetes Overview

Classification

In humans, diabetes mellitus is considered a group of metabolic diseases diagnosed by demonstrating persistent hyperglycaemia\(^1\). This hyperglycaemia can be the result of defects in insulin secretion by the beta-cells (ie. when there is beta-cell damage), insulin action, or both\(^2\). In humans, chronic hyperglycaemia leads to long term tissue damage and failure of various organs including the eyes, kidney, nerves, heart and blood vessels\(^3\). Pathogenic processes include either an insulin deficiency caused by autoimmune destruction of beta-cells in the pancreas (type 1) or insulin resistance compounded by beta-cell failure as a result of incompletely understood mechanisms (type 2)\(^4\). The classification of diabetes mellitus in humans is now based on aetiology rather than treatment, as was used in the past\(^2\).

Type 1 diabetes

Type 1 diabetes results from cellular-mediated autoimmune destruction of beta-cells of the pancreas resulting in reduced secretion and an absolute deficiency of insulin\(^4\). Autoantibodies to islet cells, insulin, glutamic acid decarboxylase and tyrosine phosphatase have been identified, and one or more are present in 85-90\% of individuals affected by the disease\(^1\).

Type 2 diabetes in humans

Type 2 diabetes accounts for about 90-95\% diabetic cases\(^3\) and involves resistance to insulin action and, at least initially, a relative (compensatory) rather than absolute decrease in insulin secretion\(^5\). Type 2 diabetes occurs when insulin sensitivity decreases along with impaired
insulin secretion due to beta-cell failure\textsuperscript{6,7}. Reduced insulin action on target tissues results in abnormal carbohydrate, fat, and protein metabolism\textsuperscript{8,9}. It can sometimes be difficult to know whether the hyperglycaemia is caused by reduced insulin secretion and/or reduced tissue response, but ultimately there is a resultant beta-cell failure\textsuperscript{2,3,5}. In human patients, one set of criteria for the diagnosis of diabetes includes the presence of one of the following three indicators, repeated on two different days\textsuperscript{5}:

1. Symptoms of diabetes plus a casual plasma glucose concentration $> 11.1$ mmol/L.
   Casual is defined as ‘any time of day without regard to time since last meal’
2. A fasting plasma glucose $> 7.0$ mmol/L\textsuperscript{10}. Fasted is defined as no caloric intake for at least 8 hours
3. A 2-h plasma glucose $7.8 < 11.0$ mmol/L following an oral GTT (an oral glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water). This diagnostic procedure is used if the casual or fasting results are abnormal, and to screen for gestational diabetes\textsuperscript{2,3}.

Overt clinical symptoms of diabetes include polyuria, polydipsia, weight loss, polyphagia and occasionally blurred vision, impaired growth and increased susceptibility to infections\textsuperscript{10}. Long-term complications resulting from microvascular and macrovascular degeneration include retinopathy, which often results in blindness, nephropathy resulting in renal failure, peripheral neuropathy (ulcers and possible need for limb amputation) and autonomic neuropathy affecting the gastrointestinal, genitourinary, and cardiovascular systems\textsuperscript{3,5,11}. Histopathological findings in type 2 diabetes in humans include concurrent loss of beta-cells and deposition of islet amyloid derived from islet amyloid polypeptide\textsuperscript{12}. This suggests that the amyloid deposition could play a role in functional beta-cell dysfunction in humans\textsuperscript{12-15}.
Epidemiological and laboratory evidence suggest that both the increased insulin resistance and the failing beta-cell response have genetic and environmental components. In fact, the genes associated with insulin resistance and metabolic syndrome in humans are closely associated with genes of lipid metabolism. In human type 2 diabetes, most individuals are obese or have more body fat around the abdominal region, and obesity is the most common cause of acquired insulin resistance. The relative risk for IGT, defined as 2-h glucose levels of 7.8 to 11.0 mmol/l on the 75-g oral glucose tolerance test, is increased more than fourfold in obese subjects compared with normal-weight subjects, yet only 25% of subjects with IGT are obese. Risk factors also include increased age, a lack of physical activity, and a genetic predisposition. Human subjects with IGT more frequently have first-degree relatives with diabetes compared to subjects with normal glucose tolerance, but >70% of humans with IGT report no heredity background of diabetes.

Glucose is the primary regulator of postprandial insulin secretion which is controlled by nutritional and hormonal signals in humans and animals. Glucagon-like peptide-1 (GLP-1), a peptide hormone produced in the intestinal epithelial endocrine L-cells, free fatty acids, and amino acids increase this effect once the blood glucose concentration reaches a threshold, and are considered insulin secretagogues. In both humans and animals, the progression from an obese insulin-resistant state to clinical type 2 diabetes is caused by beta-cell failure. The first acute phase of insulin release occurs approximately 10 minutes after glucose stimulation and the second phase of insulin secretion, which is more sustained, follows within 30 minutes and plateaus in 2 to 3 hours. The first-phase insulin secretion is defective initially in impaired fasting glucose along with a deficiency in the ability of glucose to potentiate insulin secretagogues. As hyperglycaemia progresses, beta-cell failure also progresses. By the time the fasting blood glucose concentration is above the 7.8
mmol/L, there is a 75% loss of beta-cell function

**Gestational Diabetes in humans**

Gestational diabetes mellitus is defined as any degree of glucose intolerance diagnosed during pregnancy, irrespective of whether the condition is treated with insulin or with dietary modification and even if it is not controlled after pregnancy. A fasting plasma glucose level of >7.0 mmol/L or a casual plasma glucose >11.1 mmol/L on a subsequent day followed by a 1-hr and/or 2-hr plasma glucose concentrations of 10.0 mmol/L and 8.6 mmol/L, respectively during an oGTT with a 75g glucose load. Gestational diabetes has not been reported in cats.

**Type 2 diabetes in cats**

Feline diabetes mellitus shares many similarities to human type 2 diabetes. More than 80% of cats with diabetes have clinical characteristics (polyuria, polydipsia, and polyphagia) and histologic abnormalities (such as amyloid accumulation in beta-cells) consistent with type 2 diabetes in humans. The typical onset for diabetes in cats and humans is middle-age or later. Over 50% of diabetic cats were over 10 years old and age was identified as the most important single risk factor in the development of diabetes in cats. The reported incidence of diabetes in cats varies from one in 50 to one in 400 cats, depending on the population studied. As in humans, the main preventable predisposing factors for the development of diabetes in cats are unlimited access to a nutrient dense diet leading to obesity, and a sedentary lifestyle, and the prevalence of obesity and diabetes is increasing concomitantly. Obese cats are 3.9 times more likely to develop diabetes mellitus than lean cats.
Neutered cats have nearly twice the risk and male cats have 1.5 times the risk of developing diabetes\textsuperscript{14,45,52}. Diabetes in cats, as in humans, appears to be associated with diseases, pharmacological agents, and hormones that impair peripheral tissue insulin sensitivity, such as acromegaly or hyperadrenocorticisim (80% of cats with hyperadrenocorticism develop diabetes mellitus\textsuperscript{53}), hyperthyroidism or treatment with corticosteroids or progestins\textsuperscript{46}.

Obesity is a major risk factor in the development of diabetes and thus the aim of dietary management should include weight loss\textsuperscript{40,54}. It is reported that cats at high risk of developing diabetes (ie. sedentary neutered indoor cats) should not be fed \textit{ad libitum}\textsuperscript{40,55,56} but rather given rations to ensure controlled consumption of calories. Added water in canned foods not only increases hydration but more importantly increases food volume thus increasing satiety, and the effect is reported to be greater than high fibre dry foods\textsuperscript{57}. A study in the U.K. reported that cats fed only wet food were 3 times more likely and cats fed only dry food 2 times more likely to develop diabetes than cats fed a mix of wet and dry food\textsuperscript{58} whereas another study reported that whether cats are fed wet or dry food made no difference to the risk\textsuperscript{59}. However, the latter study had a limited number of cats which may have limited the power.

\textit{Breed as a risk factor for feline diabetes}

Inbreeding in cats over 20 years has led to a number of genetic-related disorders\textsuperscript{60} and there is evidence to support genetic predisposition to diabetes in cats\textsuperscript{61}. Burmese cats from the UK, Sweden, Australia and New Zealand are now almost four times more likely to develop type 2 diabetes mellitus than other cats\textsuperscript{47,62-64}, with one in 50 of the breed in the UK being affected\textsuperscript{58}. Lean Burmese cats have been reported to have peripheral blood leukocyte gene expression, namely increased expression of ATP citrate lyase and fatty acid synthase, similar to age and
gender matched obese domestic cats. In fact, lean Burmese cats were found to have double the VLDL cholesterol level to that of obese domestic cats indicating lipid, especially triglyceride, dysregulation. A preliminary study of genetic predisposition of Burmese cats to diabetes reported a likely involvement of a major locus with a significant risk allele prevalence which was likely autosomal rather than sex-linked.

Pathophysiology of beta-cell failure in humans and cats

The most important common pathophysiologic features of type 2 diabetes in cats and humans are obesity-induced insulin-resistance, loss of beta-cell mass, and amyloid deposition in pancreatic islets. In addition to amyloid, the cat pancreas is extremely sensitive to the toxic effects of hyperglycaemia. Indeed, within 2 days of a hyperglycaemic clamp (25 to 30 mmol/L), hyperglycaemia in healthy cats induces a marked reduction of insulin secretory capacity, and subsequently a 50% decrease in beta-cells per pancreatic area. The cat is an excellent model for studying the pathophysiology of islet amyloid, as it is one of few species (human, macaque, and wild carnivores including jaguar and the African spotted leopard) to develop amyloid deposits with type 2 diabetes. In one study, 3 of 9 cats with impaired glucose tolerance and a significantly reduced first phase of insulin secretion had islet amyloid deposition, whereas 6 of the 7 diabetic cats, with significantly decreased insulin release during the first 45 min of the IVGTT compared to healthy or glucose-intolerant cats, had islet amyloid. However, there was no association between the severity of islet dysfunction and amyloid deposition and in one study, 10% of healthy cats were reported to have amyloid deposits equal to more than 50% of the islet volume indicating that the theory of amyloid deposition as a cause of progression to type 2 diabetes may not be valid. Also reported is that islet amyloid formation induces oxidative stress, which in the short term does not mediate beta cell apoptosis, but in the longer term may
feedback to further exacerbate amyloid formation and contribute to beta cell apoptosis\textsuperscript{76}. Recently, studies have suggested that only the early stages of fibril assembly, and in particular small islet amyloid oligomers, are responsible for beta cell cytotoxicity\textsuperscript{77}.

The mechanisms which result in beta-cell failure are incompletely understood\textsuperscript{78}. Originally, it was hypothesized that increased insulin secretion was associated with decreasing insulin sensitivity until it reached a point when beta-cells are unable to increase insulin production further\textsuperscript{79} and was referred to as beta-cell exhaustion. However, obesity independently can have this effect and obesity does not necessarily lead to diabetes.

The accumulation of amyloid in beta-cell islets has been hypothesized to play a role in beta-cell failure\textsuperscript{13}. This theory has been supported by reports that amyloid is present in type 2 diabetes in both humans and cats\textsuperscript{14,80} and experimentally- induced diabetes results in amyloid accumulation in the islets\textsuperscript{15}. Amyloid is an extracellular accumulation of proteinaceous substance (from the precursor amylin) which refolds forming beta-pleated sheets or fibrils in cats and humans but not in dogs, leading to loss of beta-cell mass. These fibrils are highly toxic to the beta- cells. However, this theory has also been rejected for several reasons including that amyloid accumulates not only in the beta-cells but also the alpha and gamma cells, however they seem to function normally\textsuperscript{81,82} and also that some individuals have amyloid accumulation but normal beta-cell function\textsuperscript{15,75,83}.

Amylin is a hormone and precursor of amyloid, but is produced (along with insulin) only by beta-cells and modulates insulin action\textsuperscript{79}. There is progressive accumulation of folded beta pleated sheets of intracellular amylin fibrils within the beta-cells. The misfolded amylin oligomers form toxic intracellular fibrils in the endoplasmic reticulum, leading to
programmed cell death (apoptosis) when misfolded protein is detected\textsuperscript{8}. Models of marked insulin resistance that lead to increased amylin secretion have been shown to increase amyloid deposition in cats\textsuperscript{15}. As insulin and amylin are cosecreted, it is not clear why individuals with insulin resistance and hyperinsulinaemia and thus high amylin secretion do not always form toxic amylin oligomers. It has been reported that hyperamylininaemia may then lead to reduced insulin secretion and insulin resistance, and feline diabetes\textsuperscript{80,84}. Of the numerous mechanisms resulting in beta-cell failure, islet amyloid deposition and toxic amylin oligomers may help explain species differences in the development of type 2 diabetes.

Another theory for beta-cell failure is glucose toxicity, which has been defined as ‘nonphysiological and potentially irreversible beta-cell damage caused by chronic exposure to supraphysiological glucose concentrations’\textsuperscript{85}. In humans, it is postulated that glucose toxicity can cause progressive impairment in insulin secretion and possibly result in insulin resistance\textsuperscript{86}. Cats are susceptible to glucose toxicity at high concentrations (2 days of a hyperglycaemic clamp (25 to 30 mmol/L)), which is thought to cause changes in the microenvironment of the endoplasmic reticulum and cause beta-cell death\textsuperscript{71,87-89}. However, to have hyperglycaemia initially would require a pre-existing impaired insulin response or production, thus negating this theory as the sole cause of beta-cell failure.

Another valid theory for beta-cell death is the effect of reactive oxygen species which are produced in the cell when there is excessive glucose and/or fatty acids\textsuperscript{90}. Insulin normally controls glucose homeostasis by stimulating glucose uptake into peripheral tissues and suppresses the release of stored lipids from adipose tissue\textsuperscript{26,34}. Insulin deficiency leads to dyslipidaemia, and dysfunctional metabolism of fatty acids, triglycerides and lipoproteins\textsuperscript{22,23,91}. These chronic increases in circulating glucose and lipid levels can further
impair insulin secretion and action, and cause other forms of tissue damage\textsuperscript{6,34,90}. This is also referred to as glucolipotoxicity, and is thought to cause beta-cell toxicity via increased electrochemical gradients across the mitochondrial membrane caused by oxidation of intracellular glucose and fatty acids\textsuperscript{92}. Oxidative stress leads to beta-cell apoptosis. In diabetic humans, chronic hyperglycaemia leads to further production of reactive oxygen species and deterioration of beta-cell function, which increases circulating fatty acids and the severity of the type 2 diabetes\textsuperscript{93}. This theory, however, does not explain why cats develop type 2 diabetes and dogs do not, unlike the fibrils of amylin theory. To date, literature on the relationship of reactive oxygen species and diabetes in cats is lacking.

A further theory is the possibility of an autoimmune response causing the expression of inflammatory mediators such as cytokines and chemokines (for example TNF-\textalpha and IL-1) leading to an inflammatory cascade in response to a nutrient overload which could trigger beta-cell apoptosis\textsuperscript{94,95}. These mechanisms leading to beta-cell destruction have not been studied in cats.

**Type 2 Prediabetes**

The development of overt diabetes is often preceded for months to years by a subclinical condition, prediabetes, which induces changes in target tissues. The terms impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) refer to a stage intermediate between normal glucose homeostasis and diabetes, now referred to as prediabetes\textsuperscript{3,5}. Patients with IGT and IFG do not fulfil the criteria for diagnosis of diabetes and usually return to reasonable glycaemic control with weight loss, dietary management, and exercise. Humans with fasting glucose levels \( \geq 6.1 \text{ mmol/L} \) but \(<7.0 \text{ mmol/L} \) are considered to have IFG\textsuperscript{2}. A variable but relatively high proportion (25–75\%) of people with IGT at a first test will have a normal
response at the second\textsuperscript{16}. Humans with prediabetes are at increased diabetic risk\textsuperscript{17,18,30,96}. There is no literature on the progression of IFG and IGT in cats to clinical diabetes. However, cats in diabetic remission \(n = 20\) were more likely to relapse if: 1. the fasting blood glucose concentrations were \(\geq 7.5\) to \(9.9\) mmol/L or, 2. a blood glucose concentration at 5 hours after an IVGTT was more than \(6.5\) mmol/L, than cats with lower glucose values or shorter return to baseline. In this study, the unadjusted odds ratios (OR) for potential predictors of relapse by 9 months after baseline testing in diabetic cats in remission were 12.8 and 15.2 mmol/L respectively\textsuperscript{97}.

Insulin resistance is also present in most human and feline diabetic patients\textsuperscript{7,34,39,87,98}. Insulin levels increase to compensate for the resistance of muscle, fat, liver and pancreas to the effects of insulin\textsuperscript{8}. A higher plasma insulin concentration is required to produce the same glucose uptake into tissues compared with when insulin sensitivity is normal\textsuperscript{40}. Decompensation and absolute hypoinsulinaemia occurs well before the renal threshold for glucose is exceeded (16 mmol/L in cats)\textsuperscript{46}.

Insulin resistance develops as a consequence of the effects of inflammatory and hormonal factors\textsuperscript{7}, endoplasmic reticulum stress (by chronic exposure to excessive nutrients and dysfunctional protein kinases), and glucolipotoxicity induced by beta- cell dysfunction\textsuperscript{34,92}. In both animals and humans, the triggering factor for transition from an obese, insulin-resistant state to overt type 2 diabetes is beta-cell failure, which involves both a partial loss of beta-cell mass and a deterioration of beta-cell function\textsuperscript{8,19,99}. However, obese and insulin-resistant humans can remain in a state of beta-cell compensation, but later some individuals progress to beta cell failure and diabetes\textsuperscript{34}. Obese patients with fasting hyperglycaemia all have abnormal islet function\textsuperscript{8}. Failure of the beta-cells to release insulin appropriately is a
common abnormality between human type 2 diabetes\textsuperscript{100} and feline diabetes\textsuperscript{101}.

Using the euglycaemic hyperinsulinaemic clamp-an accepted measure of insulin sensitivity\textsuperscript{102}-obese cats have been shown to be markedly insulin resistant\textsuperscript{54,102,103}. A 50\% increase in body mass index led to a 60\% decrease in insulin sensitivity and at basal insulin concentrations, there was a decreased ability to regulate glucose production by the liver\textsuperscript{103}. Obesity also leads to changes in insulin secretion. While fasting blood glucose concentrations are still maintained within the normal range\textsuperscript{39}, the insulin secretion pattern has already changed in obese cats compared with lean cats, there is loss of first phase insulin secretion followed by a large increase in second-phase release\textsuperscript{104}. In some individuals, prolonged insulin resistance eventually leads to a decrease in the insulin secretory capacity\textsuperscript{15}.

One study in the U.K. screened 11,858 people through a survey sent to a census population, and used interview data and oral glucose tolerance testing. Based on the data, it was estimated that as many as 50\% of people in the UK were undiagnosed diabetics\textsuperscript{105}. Consistent with other studies, one-third to one-half of people in U.S. populations over 20 years of age with type 2 diabetes are undiagnosed and, hence, untreated\textsuperscript{11,25,105,106}.

**Diet as a risk factor for feline diabetes**

Cats are obligate carnivores and their natural diet is protein-rich prey\textsuperscript{59,107}. Cats have a reduced adaptation to managing a carbohydrate load including low glucokinase activity in the liver and muscle\textsuperscript{108,109} which limits their ability to metabolize large amounts of carbohydrate\textsuperscript{110}. They also have a significantly higher protein requirement than other mammals and require the protein to be mainly from an animal source to supply essential amino acids\textsuperscript{111}. However, with domestication they have become more sedentary animals
eating a more energy dense diet, higher in carbohydrates\textsuperscript{59}.

Fat is an essential nutrient in a cat’s diet, however, a high fat diet (50\% ME) can promote obesity\textsuperscript{112,113}, increase insulin concentrations postprandially\textsuperscript{113}, slow glucose clearance and a decrease acute insulin response\textsuperscript{114} and obesity is a major risk factor for feline diabetes\textsuperscript{46}. It is frequently reported that obesity and type 2 diabetes can be influenced by a variety of factors which include diet and management, and it is clear that the interaction of the different nutrients has a confounding effect on many of the results reported\textsuperscript{46,85,107,112}.

The effects of carbohydrate, fat, and protein on glucose metabolism in healthy cats has been studied extensively\textsuperscript{115-120}. However, due to the lack of standardization of nutrient content of the diets, the short timespan over which the studies were performed and the lack of standardization of the length of fasting prior to initial sampling it makes comparison results between studies difficult. For example, numerous studies reporting the effects of carbohydrate on blood glucose concentrations used diets containing carbohydrate ranging from <25\% ME to > 50\% ME\textsuperscript{115,121-124}. Additionally, studies have shown that it can take over 8 hours to reach the postprandial peak blood glucose concentration and 12 to 24 hours for blood glucose concentration to return to fasting levels after a single meal\textsuperscript{110,116,118,125}.

Persistent postprandial hyperglycaemia is detrimental to beta-cell function and it is the carbohydrate and not protein content of the diet which determines the postprandial glucose concentration\textsuperscript{123,126,127}. Therefore, a low carbohydrate diet is likely beneficial to reduce postprandial hyperglycaemia and therefore decrease the risk of developing diabetes such as in cats with IFG or IGT\textsuperscript{54,123,128}. In fact, it has been reported that cats on a high carbohydrate diet, the mean postprandial blood glucose concentration over the entire 24-h period was 8-9
mmol/L\textsuperscript{117} and after moderate weight gain reached 13.4 mmol/L in some cats.

Diets lower in carbohydrate and higher in protein are associated with a lower postprandial blood glucose and insulin concentration\textsuperscript{129-131}. One study reported that cats on a high carbohydrate (51\% ME) low protein (21\%) diet had higher postprandial blood glucose and insulin concentrations compared to a low carbohydrate (23\%) high protein (47\%) diet but the low carbohydrate diet had higher fat content which promoted greater weight gain when fed \textit{ad libitum}\textsuperscript{117}. A recent study of 63 diabetic cats found that low carbohydrate (12\% ME) and low fibre (0.1 g/100g as fed) diet was more likely to lead to remission than a moderate carbohydrate (26\% ME) and high fibre diet (3.1 g/100g as fed)\textsuperscript{132}.

In one study, the energy density of dry food diets was not significantly associated with the development of diabetes mellitus, whereas both indoor confinement and low physical activity were in data from cats collected via a questionnaire (n=96 diabetic, n=192 matched controls)\textsuperscript{59}. However, questionnaire data can be unreliable\textsuperscript{133}. In overweight cats, source of dietary carbohydrate has been reported to have an effect on blood glucose concentration, insulin secretion and food intake\textsuperscript{124}. A mixture of sorghum and corn was reported to lead to less glucose intolerance and insulin resistance than rice and may thus decrease the risk of diabetes\textsuperscript{124}. However, the quantity of food eaten was not discussed.

**Diet for treatment of feline diabetes**

Dietary management is important in managing a cat at risk of developing diabetes. The aims should include to minimize stress on the beta-cells to produce insulin\textsuperscript{134}, weight control\textsuperscript{40}, and minimizing postprandial hyperglycaemia and fluctuations in blood glucose concentration\textsuperscript{124,135}. Studies show that a diet high in protein (>40\% ME) and very low in
carbohydrate (<15% ME) is the most effective to achieve the aims related to glucose and insulin homeostasis. A high protein diet also helps maintain muscle mass during weight loss and assists in preventing hepatic lipidosis, normalizing insulin function and increasing metabolism. However, the high fat content of commercial high protein, low carbohydrate diets can promote weight gain because weight gain increases with increasing fat content. Another study reported that diabetic cats (n=6) on twice daily glargine and fed a low carbohydrate (3.34g/ 100 kcal), high protein diet (13.88g/ 100 kcal), when compared with controls (n=6) showed no difference in the control of diabetes.

**Diagnosis of diabetes in cats**

Clinical signs in some cats are subtle initially and progressive over months to years. These cats may show none of the classic signs until progressive beta-cell failure impairs insulin secretion to the point that hyperglycaemia exceeds the renal threshold (16 mmol/L). At this point, before the classic signs of polyuria, polydipsia, polyphagia, muscle wasting, neuropathy especially in the hindlimbs and weight loss are seen, gastrointestinal signs such as vomiting may develop. Diseases which are reported to occur concurrently at the time of diagnosis include hyperthyroidism, inflammatory bowel disease, eosinophilic granuloma complex, anaemia, neoplasia, and renal failure, however what must be considered is that older cats are overrepresented, and they are also at higher risk of developing these diseases.

A diagnosis of diabetes mellitus is made when the appropriate clinical signs are evident, along with a persistent hyperglycaemia which is typically above 16 mmol/L (the renal threshold in cats) and glucosuria. When these cats present at clinics, a thorough history, physical examination, complete blood count, serum biochemistry, and urinalysis should be measured. Cats with clinical diabetes will need treatment with insulin and dietary and
weight management to control the disease and to increase the likelihood of diabetic remission.\textsuperscript{139,144-146}

In general, lithium heparinised plasma samples analysed by automated chemical analysers using either hexokinase or glucose oxidase methods are considered the gold standard for determining an animal’s venous blood glucose concentration.\textsuperscript{147,148} Upper limits for normal blood glucose concentration in cats cited in established texts range from 6.1 mmol/L\textsuperscript{149} to 7.3 mmol/L.\textsuperscript{150,151} Idexx Laboratories, a major veterinary diagnostic laboratory, have a reference range of blood glucose of 3.2-7.5 mmol/L in healthy cats. This was established by analysis of patient data (filtered) from mid-1990 and rechecked against patient data (filtered) from 2006-07 (Dr. John Mackie, personal communication). The 2006-07 analysis of 2,862 samples is potentially biased at the upper end because of the failure to rigorously exclude non-fasted or stressed cats. However, the upper limit is similar to those quoted in the literature.

Stress hyperglycaemia is a complicating factor when interpreting blood glucose concentrations in cats.\textsuperscript{152,153} Struggling scores and lactate concentrations were predictive of stress hyperglycaemia in a study of 20 healthy adult cats given a 5-minute spray bath. Blood glucose of individual cats increased from a baseline of 4.6 mmol/L to 10.8 mmol/L.\textsuperscript{153} Stress hyperglycaemia persists for at least 90–120 minutes in healthy cats that have been restrained or exposed to other types of short-term stress.\textsuperscript{153} In a 4-year retrospective study of 2278 sick cats at initial presentation at a veterinary hospital, 1388 cats (61\%) had normal blood glucose concentrations, 827 cats (36\%) were hyperglycaemic (blood glucose > 8 mmol/l) and, 63 cats (3\%) were hypoglycaemic.\textsuperscript{154} Blood glucose concentrations in cats with stress hyperglycaemia were between 8.1 and 60.4 mmol/l (median 10.3 mmol/L), and in cats with diabetes mellitus between 8.5 and 70.0 mmol/L (median 27.7mmol/L).\textsuperscript{154}
Diagnosis of prediabetes in cats

There are no reports in the literature of a suitable non-stressful and relatively non-invasive screening test available for diagnosing prediabetes in client owned cats in a clinical environment. Diabetes in cats is usually only diagnosed once clinical signs are evident, ie. once the renal threshold of blood glucose has been reached\(^{46}\). These cats usually need insulin treatment\(^{61,155,156}\) which is stressful to both owner and cat. Studies have reported an increased postprandial blood glucose concentration for 12-19 hours with a diet containing 30-50% ME of carbohydrate in lean cats, 18 hours for obese cats and in some cases over 24 hours\(^{54,117,122}\), suggesting that cats should be fasted for at least 18 hours for any screening test which involves a fasting blood glucose concentration. A validated screening test for prediabetes in cats, as exists for human patients, is lacking and would improve diagnosis of prediabetes so that preventive health programs can be implemented before insulin-dependent diabetes ensues.

Glucose tolerance tests

Intravenous glucose tolerance testing has been used frequently as a research tool to measure glucose tolerance in cats, and reference intervals for parameters such as t\(^\frac{1}{2}\) and area under the curve established\(^{72,104,157}\). The classic test involves intravenous catheterization, and a bolus injection of glucose (usually 50% glucose infusion via an indwelling iv catheter) followed by 10 blood glucose measurements from an indwelling jugular catheter inserted under a general anaesthetic\(^{114,117,157}\). From a clinical perspective, these tests have been too labour intensive and invasive to be used as a routine screening method. A simplified glucose tolerance test was established\(^{158}\), where cats did not need to be anaesthetised for catheter placement before the test. Instead, 2 cephalic catheters were placed aseptically and the test
started after 3 hours. One catheter was used for the infusion of glucose and the other for
blood sampling with a portable blood glucose monitor. Reference intervals for healthy cats
were established for this methodology. To date, there are no studies reporting the
methodology and reference interval for a more simplified, potentially less stressful method
for diagnosing feline prediabetes in a clinical setting.

**Portable glucose meters**

Portable blood glucose meters are commonly used in veterinary medicine for quick and
convenient measurement of blood glucose concentration. They use only a small
amount of blood and require minimal restraint. Two methods are commonly used by
PBGM for measuring blood glucose concentration: reflectance photometric systems and
amperometric biosensor systems.

The significant benefit of portable blood glucose meters is their ability to be used by owners
for home monitoring. When investigating the feasibility of home-monitoring of
blood glucose in diabetic cats by owners, it was reported that the majority of problems with
respect to restraint and obtaining an adequate blood sample were resolved during the study.

There have been numerous studies of human portable blood glucose monitors to deem them
acceptable when used with cats including comparison to the ‘gold standard’ hexokinase
introduced a new PBGM that has been calibrated for use in cats and dogs. The AlphaTRAK
needs only a minute (0.3 uL) blood sample in comparison to most other PBGMs on the
market. The glucometer was validated by comparing the performance of the AlphaTRAK and
the Ascensia ELITE® (Bayer HealthCare, Zurich, Switzerland) which was developed for humans\textsuperscript{160}. At low blood glucose concentrations, the AlphaTRAK (Zoetis) tended to underestimate true concentrations, whereas other PBGMs arbitrarily under- or overestimated true values, which can be considered a disadvantage\textsuperscript{163}.

\textit{Capillary sampling}

Capillary blood sampling is a useful method of obtaining a drop of blood for analysis by PBGMs. The marginal ear vein technique has been successfully used in cats\textsuperscript{163} and can be used by owners for home monitoring\textsuperscript{166}, unlike venepuncture which involves more restraint and stress\textsuperscript{169}. The protocols can vary, but most methods involve warming the pinna, using a lancing device to form a blood droplet and contacting the strip of the glucose meter with the droplet to perform the analysis\textsuperscript{169}. A similar method can be used on the pad of the paw.

Venous and capillary blood samples were studied from 33 dogs, males and females, of different breeds, weights and ages\textsuperscript{161}. The results with the different types of blood were statistically not different, which implies that that venous and capillary samples can be used interchangeably. Blood glucose concentrations measured with a PBGM using blood samples obtained from a marginal ear vein nick technique, from a peripheral venous catheter, and from direct venipuncture were compared in 10 healthy and 11 diabetic cats\textsuperscript{170} and considered the marginal ear vein nick technique a reasonable alternative to venous blood collection when taking serial measurement of blood glucose concentrations in cats.

The suitability of the microlet vaculance lancing device and a PBGM for use by pet owners in cats and dogs were evaluated\textsuperscript{171}. It was reported that all dog owners (n=7) and three cat owners (n=7) were able to perform a reliable blood glucose curve of 6 blood glucose
measurements. The most frequently encountered problems were inadequate formation of a blood drop due to excessive digital pressure on the pinna, repeatedly depressing the plunger of the lancet device instead of allowing the negative pressure to slowly build up, and failure to fill the test strip adequately with blood. The authors concluded that these steps of the procedure need to be clearly explained and demonstrated to owners and that home monitoring of blood glucose concentrations is good alternative to hospitalisation. Abbott Animal Health (now part of Zoetis) has developed a lancing device which is suitable for use in cats and dogs. It does not cause a vacuum but does reliably produce a blood droplet large enough (>0.3 µL) for the AlphaTRAK PBGM.

Other indicators of abnormal glucose metabolism, insulin resistance, obesity, and diabetes

Fructosamine

Fructosamine (also known as glycated plasma proteins) is formed by the glycosylation of plasma proteins and its concentration is dependent on the mean blood glucose concentration in the 10 to 15 days preceding sampling\textsuperscript{172}. It has been used as a measure of long term glycaemic control in cats\textsuperscript{173,174} and its differentiation from transient stress hyperglycaemia\textsuperscript{175}. Body weight is positively correlated with serum fructosamine concentration, and lean cats have lower concentrations than obese cats\textsuperscript{176}. Fructosamine was reported to be a very sensitive (92\%) and specific (96\%) in differentiating non-diabetic from diabetic cats\textsuperscript{177}. However, in cats with moderate hyperglycaemia (17 mmol/L), fructosamine concentration mostly fluctuated under the upper limit of the reference range (95\% reference interval 154-331 µmol/L) during a 42-day glucose infusion. Additionally, the range of fructosamine concentrations associated with a given glucose concentration was wide, with the critical difference for fructosamine being 33 µmol/L\textsuperscript{178}. Differences in serial measurements of less
than 33 µmol/l may not be indicative of fluctuations in blood glucose measurements. Fructosamine is not suitable to use as a screening test for prediabetes in cats where blood glucose concentrations of 6.5 to 16 mmol/L are expected.

**Insulin sensitivity**

The HEC and MinMod techniques have been used to estimate insulin sensitivity in cats\(^{102,157}\). The HEC involves the infusion of insulin at a constant rate and glucose is infused to maintain blood glucose at the baseline value. The Min Mod requires 31 blood samples and a specialized computer program to calculate the insulin sensitivity index. Thus, neither of these techniques are practical in a clinical environment. The most useful predictors of insulin sensitivity in cats were basal plasma insulin concentrations after food was withheld for 12 hours and the HOMA, which is the product of basal glucose and insulin concentrations divided by 22.5\(^{179}\). Insulin sensitivity and glucose tolerance test were performed on 16 cats which were fed *ad libitum* for 9-12 months to gain weight before and after weight gain\(^{54}\). The findings of this study included an increased risk of developing impaired glucose tolerance in cats with high basal insulin concentrations, suggesting that some cats have an underlying predisposition to develop glucose intolerance as a result of intrinsic insulin resistance. It was suggested that if these cats became obese they were at higher risk of developing clinical diabetes. Through early detection of insulin resistance, early intervention programs could be introduced to aid in preventing impaired glucose tolerance and clinical diabetes. Insulin, however, cannot be measured quickly as a screening test in a clinic environment and is not routinely measured in veterinary laboratories, although five radioimmunoassay kits designed to measure human insulin concentrations were tested on feline insulin\(^{180}\) and more recently, new ELISA test kits have now become available and have been validated for feline blood by the Morinaga Institute of Biological Science, Kanagawa, Japan and Mercodia AB, Uppsala,
Sweden. None of the tested kits is available anymore.

**Feline pancreatic lipase**

Measurement of serum fPLi has been proposed as a sensitive and specific test for the detection of pancreatitis in cats. It has been hypothesised that pancreatitis induces death of pancreatic beta-cells, which could lead to the development of clinical diabetes, but this has not yet been substantiated in cats and it is unknown whether pancreatitis in cats is the trigger or the result of diabetes.

**Triglycerides**

Many (28%) Australian Burmese cats exhibit marked post-prandial hypertriglyceridaemia 4 and 6 hours after an oral fat tolerance test compared to the control cats (median 4-h triglyceride 2.8 mmol/L and 1.5 mmol/L for Burmese and control respectively, median 6-h triglyceride 8.2 mmol/L and 1.0 mmol/L for Burmese and control respectively). However, they did not have significantly different fasting insulin, fructosamine, NEFA, or lipoprotein concentrations. Burmese cats are also at increased risk of developing lipid aqueous, which includes an underlying disorder of lipid metabolism that results in increased serum triglyceride concentrations. It is unknown whether this is related to Burmese being 3-4 times more likely to develop diabetes than other breeds of cats. However, fatty acids have been reported to be toxic to beta-cells which could increase the likely progression to clinical diabetes. Obesity and type 2 diabetes in cats is associated with hypertriglyceridaemia, insulin resistance, an overproduction of VLDL in the liver, and decreased adiponectin, thus leading to a decrease in its insulin-sensitizing function. There are no studies to date that compare baseline triglycerides in Burmese cats and other cats at risk of developing diabetes to other measures of glucose tolerance.
Adipokines

Adipokines are biologically active proteins originating from adipose tissue that have local and systemic effects. The relationship between obesity and adipokines as markers of inflammation has been studied extensively in humans, dogs, and cats.

Leptin

Leptin is the best characterized adipokine in domestic animal species. The main physiologic role of leptin is to regulate body fat mass through modulating energy intake and energy metabolism. Appetite suppression occurs via binding to receptors in the hypothalamic satiety centres. Adipocytes are the main site of leptin synthesis and the main contributor to serum leptin concentrations, which increase with obesity. Other tissues such as liver, gastric mucosa, mammary glands and placenta may also secrete leptin. Serum concentration is predominantly defined by body fat mass, but transiently increases with a meal and decreases with fasting. In cats, this postprandial increase was only seen in cats after weight gain and not in lean cats. In humans, leptin has been reported to have a direct anti-diabetic effect through triglyceride depletion. In humans, the association of leptin with body composition and type 2 diabetes were studied and it was reported that fat mass rather than insulin resistance has a more significant influence on leptin fluctuations. In cats, despite high concentrations of leptin, overweight and obese cats continue to gain weight and appear to be less sensitive to the appetite suppressing action of leptin compared with cats of ideal body condition score, suggesting leptin resistance. Regardless of fat mass, insulin-resistant cats have higher circulating leptin concentrations compared with insulin sensitive cats, suggesting that hyperinsulinaemia associated with insulin resistance itself may stimulate leptin production. An ELISA test for leptin has been validated for use.
Adiponectin

Adiponectin is thought to be produced almost exclusively by mature adipocytes. Adiponectin has been investigated widely due to its association with adiposity and the metabolic syndrome in humans. It directly sensitizes the body to insulin by increasing fatty acid oxidation and decreases triglyceride content in the liver and other tissues. Unlike other adipokines, adiponectin is decreased with obesity and is a contributor to the insulin resistance of obesity in humans. Plasma adiponectin in obese cats is significantly lower than in lean controls, and may also be associated with insulin function in cats. In the circulation, adiponectin may form trimers, hexamers or high molecular weight multimers. The high molecular weight multimers have the most biological activity and are best correlated with insulin sensitivity in cats. Increases in fat mass result in decreased circulating adiponectin, whereas weight loss results in increased adiponectin concentrations. In the liver, adiponectin improves insulin sensitivity while reducing triglyceride content and gluconeogenesis. In one study of 104 lean, overweight and obese dogs, it was reported that plasma leptin concentrations after a 24-h fast were associated with obesity-associated changes in insulin sensitivity measured by HOMA and compensatory hyperinsulinaemia in obese dogs, but adiponectin did not appear to be involved. In cats, adiponectin expression was demonstrated in fat pads and expression was significantly greater in visceral compared to subcutaneous depots. In cats, associations with diet composition have been reported. Cats were randomly allocated either a low carbohydrate (19% ME; n=15) and a high carbohydrate (52% ME; n=16) diet and it was reported that a high carbohydrate diet fed at maintenance energy requirements was associated with higher fasting, but not postprandial concentrations of HMW adiponectin.
**Monocyte chemoattractant protein-1 (MCP-1)**

Circulating MCP-1 is significantly increased in patients with type 2 diabetes\(^{210-215}\). An inflammatory adipokine, MCP-1 is produced by monocytes and adipocytes and increases in concentration with obesity in humans\(^{195}\) and dogs\(^{216}\). A positive relationship between plasma levels of MCP-1 and HOMA scores has been identified in baboons\(^ {217}\) and in overweight and obese humans who are prone to having low insulin sensitivity\(^ {218}\). An increase in MCP-1 expression in adipose tissue in mice contributes to the macrophage infiltration into adipose tissue. It also impairs insulin-stimulated glucose uptake in adipocytes, resulting in insulin resistance\(^ {219}\), and hepatic steatosis associated with obesity in mice\(^ {220}\). MCP-1 protein expression was higher in omental fat than in subcutaneous fat in severely obese patients, and was associated with increased macrophage infiltration into omental fat\(^ {221}\). Similar relationships have not been reported in cats. However, the expression of MCP-1 was quantified in isolated islets of healthy cats using feline-specific real-time PCR primers nested within the mRNA sequences\(^ {222}\) and MCP-1 mRNA was detected in subcutaneous and visceral fat but not in skeletal muscle in cats\(^ {223}\).

**Section 2: Metabolomics**

**Definitions**

Metabolomics has been defined as the measurement of a comprehensive set of metabolites, excluding enzymes, structural molecules, and genetic material, in a body fluid or tissue extract using a global approach, meaning that all the metabolites are measured simultaneously\(^ {224-226}\). The metabolites which are measured include carbohydrates, lipids, peptides, and some proteins. The aim of metabolomics, using high-throughput sample analysis and software pattern recognition processes is to define a metabolic phenotype
(metabotype) in the study species\textsuperscript{226} that could determine the full metabolite profile (metabolome) of a cell, tissue, or organism\textsuperscript{227}.

Metabolomics has numerous advantages over genomics, transcriptomics, proteomics\textsuperscript{101}. Firstly, the number of molecules is smaller (n= 6500), compared to genes (n=25000), transcripts (n=100000) and proteins (n=100000)\textsuperscript{228}. Secondly, metabolites provide a more ‘whole system’ profile of biological status, measuring chemical phenotypes that are the net result of the other ‘omic’ variabilities. Thirdly, metabolomics is a powerful investigative tool that provides information on drug therapy outcomes\textsuperscript{228}.

Alterations in the metabolic profile often present much earlier in the course of disease than induced histopathological changes\textsuperscript{229,230}. Metabolomics can therefore be used as a sensitive, early indicator of a disease process. Unlike many genes and proteins, metabolites are conserved across species lines. Therefore, screening patterns can be applied to a wide range of species and so the detection methods and equipment used in one organism can easily be applied to another without the need for recalibration\textsuperscript{224,226}. This versatility means that metabolomics-based approaches can be applied to studies in a wide variety of disciplines\textsuperscript{226}. Other advantages of studying metabolites include cost-effectiveness and rapid measurements. These features enable large numbers of samples to be processed quickly, thereby providing a high-throughput analytical tool.

Type 2 diabetes, obesity, and metabolic syndrome are all disorders involving multiple organ systems and to interpret the biochemical changes that occur in one organ it is necessary to understand how these interact in the entire body system. In this respect, by sampling a tissue/blood or urine for multiple markers of a disease state, it is possible to monitor changes at the
whole animal level\textsuperscript{231}.

**Diagnostic applications**

Metabolomics can be either targeted (where metabolites are identified and exact concentrations measured), or non-targeted (where two biological states are compared by simultaneously measuring as many metabolites as possible in a biological specimen), often referred to as the global approach\textsuperscript{231}.

Non-targeted metabolomics technologies have the potential for providing novel biomarkers of disease and drug efficacy, and are increasingly being incorporated into biomarker exploration studies\textsuperscript{232}. Biomarkers have been defined as ‘a characteristic that is objectively measured and evaluated as an indicator of normal biological processes of pharmacological responses to therapeutic intervention’\textsuperscript{233}. The aim is to find a metabolite or groups of metabolites most representative of the condition evaluated\textsuperscript{224}; multiple markers may be of greater diagnostic value than single ones\textsuperscript{234}. For example, using pattern recognition methods\textsuperscript{235,236}, biomarkers for neurodegeneration associated with Alzheimer disease have been studied to improve the clinical diagnosis from 10-15\%\textsuperscript{237}, to assess disease severity and in the future, commence treatment when it is most effective in the early stages of the disease\textsuperscript{238}. Mass spectrometry has been used to identify biomarkers for women’s cancers (breast, ovarian and cervical) in urine\textsuperscript{239}, capillary gas chromatography–mass spectrometry for biomarkers in lung cancer\textsuperscript{240}. NMR for prostatic cancer in prostatic fluid and tissues in humans\textsuperscript{241}. Numerous other studies report the identification of potential biomarkers in cardiovascular disease in humans\textsuperscript{242-244} and dogs\textsuperscript{234}, predictive biomarkers of liver dysfunction in humans when patients are deprived of choline\textsuperscript{245} and fatty liver disease\textsuperscript{246}, and toxicity studies such as with herbicides in rats\textsuperscript{247}.
The three criteria for an ideal biomarker (or -markers) for cardiovascular disease in humans has been discussed but are applicable to any disease in human or veterinary medicine: 1. Can the clinician measure it (ie. following biomarker discovery, a targeted approach is necessary)? 2. Does it add new information (ie. the need for external validation)? and 3. Does it help the clinician to manage the patients (ie. early detection of subclinical disease, assessment of risk, early treatment of disease and monitoring of therapeutic success)? Novel biomarkers that better reflect insulin resistance and/or beta-cell function may help identify patients who are, more or less, likely to respond to the various current, as well as future anti-diabetic pharmaceutical products. A targeted approach where a number of predefined metabolites are measured would allow for monitoring treatment success and disease progression.

Technologies used

Sample Preparation and metabolite extraction

Sample preparation is a crucial step in any metabolomics assay to ensure preservation of the molecules studied. This is dependent on the type of sample being used (tissue, cell or fluid), the degree of fractionation necessary, where the sample is gel based or free and whether mass spectrometry, needing filtration, will be applied. For example, mouse liver samples for GC x GC-MS analysis were freeze-dried overnight and homogenized and solid-phase microextraction was used for studying lung cancers and other volatile disease markers in blood. The methanol–chloroform technique enables extraction of both aqueous metabolites and lipids simultaneously to allow, for example, numerous brain pathologies in which both aqueous metabolite and lipid changes occur to be studied simultaneously. Using muscle and liver tissues from fish, homogenous tissues were extracted using perchloric...
acid, acetonitrile/water, methanol/water, and methanol/chloroform/water. It was stated that single organic solvent extractions are quick and easy and produce reasonable results but they concluded that the methanol/chloroform/water extraction is the preferred method due to its superior results with both hydrophilic hydrophobic metabolites.

**Chemical Analysis via platforms**

Metabolomics can be performed using individual biochemical assays based on enzymatic reactions, but the cost and time involved would make it impractical to be used routinely. Griffin and colleagues state that ‘metabolomics offers a conceptual leap forward in measuring as many metabolites as possible in one assay’. However, no single analytical approach is sufficient to cover the whole metabolome, because of the wide range of concentrations, masses and polarities. Thus, a combination of analytical platforms is often used.

Nuclear magnetic resonance, mass spectrometry, the latter often in combination with either gas or liquid chromatography are the technologies used. Nuclear magnetic resonance has been the main technique used in metabolomics and involves the application of strong magnetic resonance fields and radio-frequency pulses to the nuclei of atoms which change from a low energy to high energy state and the radiation emitted once they relax back to the low energy state is detected. Little or no sample preparation is required for NMR, is rapid and provides highly reproducible results (CV 1-2%) with up to 200 samples measured within a day. Since NMR spectroscopy is a very reproducible technique it is well suited as a diagnostic tool in a clinical setting. A disadvantage, however, is that NMR lacks the sensitivity of MS.

Mass spectrometry involves firstly ionization of the metabolite and then separation of the
ions using an electromagnetic field\textsuperscript{255}. It provides high sensitivity but lacks reproducibility\textsuperscript{229}. When used for screening the metabolome therefore not readily applicable in clinical analysis. However, MS offers possibilities for developing robust methods for quantifying metabolites in a biomarker profile\textsuperscript{256,259}. Mass spectrometry is usually combined for better analytical separation of the metabolites. The two MS and NMR methods used in parallel complement each other to ensure better biomarker identification and validation\textsuperscript{260}. For example, combining LC and NMR does increase sensitivity but still is unlikely to reach the levels provided by MS.

Gas chromatography coupled to mass spectrometry is a highly suitable technique for metabolomics analyses due to the high separation power, reproducible retention times, sensitive, selective-mass detection, and established databases. After isolating a sample, derivatives are prepared then gas chromatography is used for separations and mass spectrometry for identification\textsuperscript{226,261}. It is often considered the ‘gold standard’ in metabolomics\textsuperscript{226,262,263}. Many of the metabolites detected by GC-MS are involved in amino acid metabolism\textsuperscript{263}, the Krebs cycle\textsuperscript{100}, glycolysis\textsuperscript{263} and beta-oxidation\textsuperscript{231}. Disadvantages include the increased processing and analysis time needed per sample and the fact that structural isomers are sometimes difficult to differentiate\textsuperscript{254}.

A new two-dimensional technique has been reported, GC x GC–MS, has higher chromatographic separation power, a broader dynamic range and lower detection limits than GC-MS, and it was suggested that it be the preferred technique for metabolomics analysis. However, quantification of metabolites in samples using GC x GC–MS is a challenge\textsuperscript{249}.

In drug development studies LC-MS shows great promise, is more sensitive than NMR, and
has the added advantage over GC-MS in that sample volatility is not needed\textsuperscript{264}. It is thus potentially a universal technique\textsuperscript{226,265,266}. However, because several different ionization techniques are in use spectral libraries are not as available and there is poor reproducibility of results\textsuperscript{226}.

\textit{Interpretation: pattern recognition and data processing}

Scaling and pre-processing can vary results significantly\textsuperscript{256}. In metabolomics experiments, the biological variation of interest is inevitably confounded with unwanted variation, often due to both the unwanted experimental and unwanted biological variability (confounding factors) which may be difficult to quantify and differentiate from the wanted variation (ie. the biological factors of interest being studied)\textsuperscript{267}. The first step involves identifying overall sources of variation and the second step involves normalizing (either removing the overall unwanted variation component or accommodating it in an appropriate statistical model). The third step involves assessing the normalizing method.

Regardless of method chosen, both statistical and biological variations are critical for accurate analysis of data\textsuperscript{256}. Commonly used approaches to handling unwanted variation include:

1. Scaling factors, which assign an appropriate weight to each sample attempting to make them comparable\textsuperscript{267-269}. The disadvantage is that when one group of metabolites is increased in abundances then this method automatically decreases other groups of metabolites.

2. Use of quality control samples, which are composed of identical amounts of metabolites which are supposedly representative of those of the biological samples\textsuperscript{267,270}. However, there may be preparation issues in large scale studies and
cannot remove unwanted biological variability.

3. Use of internal standards: these are known metabolites added to the sample before extraction\textsuperscript{267}. In single internal standard, the log metabolite abundance of a single internal standard is subtracted from the log abundances of the metabolites in each sample. This can lead to highly variable normalized values, which depend on the compound that is used as the internal standard\textsuperscript{271}. In multiple internal standards, two methods are commonly used. Average internal standard, involves subtracting the average of the multiple internal standards from the log abundances of the metabolites individually. Normalization using optimal selection of multiple internal standards\textsuperscript{268} selecting the optimal combination of multiple internal standards using multiple linear regression.

4. Use of quality control metabolites involves using metabolites in the biological samples which are unassociated with the wanted variation (factors of interest)\textsuperscript{268}, allowing the unwanted variation to be accommodated.

**Statistical analysis of metabolomic data**

*Visualization of the wanted and unwanted variation*

One of the main aims of metabolomics research is classifying groups of individuals based on their metabolic profile\textsuperscript{272}. Metabolomics involves analysing a large number of biological samples and extremely complex datasets, often with considerable overlaps. Thus, univariate statistics such as Student’s t-test are impractical due to the sheer volume of data and the inability to correlate numerous variables at once\textsuperscript{226}. Multivariate methods are invariably always used in metabolomics analyses because one biomarker is usually not sufficiently specific for a condition and all variables in a dataset can be considered simultaneously\textsuperscript{226}. These multivariate statistical techniques are categorized as either unsupervised (where values
are assessed without knowing the class into which the metabolites belong) or supervised (i.e., control and diseased animals, where there is prior knowledge of class membership). The latter allows maximal separation of groups studied but care must be taken not to ‘overfit’ the data. To avoid this, cross-validation with supervised methods is always recommended226,256. A combination of multivariate statistics and targeted univariate techniques are likely to yield the best results226 for pattern recognition273.

Relative log abundance plots are often used as the first step in visualizing metabolomics data271. The median of each metabolite in the data matrix within (for within group RLA plot) or across (for across group RLA plot) the factors of interest are computed and then the median subtracted from each metabolite. Box plots of this centred data matrix are presented as the RLA plots. Within group RLA plots should have a median close to zero and low variation around the median which is used to assess the tightness of the replicates274.

A volcano plot is a type of scatter-plot that is used to quickly identify changes in large data sets composed of replicate data267 by plotting the negative log of the P-value on the y axis (usually base 10). This results in data points with low P-values appearing toward the top of the plot. Volcano plots can be used as a means of visualizing differentially abundant metabolites which deviate markedly from the rest274.

Principal component analysis is a statistical procedure that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables called principal components or clusters within multivariate data275-277. It gives an overview of the dataset, ie. does it contain groups, are there any outliers and can a single calibration model be fitted or are separate models required.
PCA can be performed on sample subgroups or across the whole dataset. The former allow easier detection of outliers and time trends and the latter whether there are any undesirable qualities in the data\textsuperscript{275,278}.

Another group of multivariate techniques which have been used to visualize the biological factors of interest and also the factors of unwanted variation is hierarchical cluster analysis\textsuperscript{279}, which is used to sequentially pair variables, clusters, or variables and clusters to build a hierarchy of clusters. The clusters and unclustered variables are tried in all possible pairs, and the pair producing the highest average inter-correlation within the trial cluster is chosen as the new cluster\textsuperscript{280}.

The orthogonal signal correction method is a technique used for pre-processing in various analytical platforms including NIR-spectra used in NMR before they are subjected to a multivariate calibration\textsuperscript{281}. The method filters the data to allow pattern recognition without loss of the measured response variable (factors of interest), while eliminating unwanted variation. Results of a study comparing OSC with other processing methods as well as no pre-processing and reported that OSC gave substantial improvements to the results showed that OSC is the only effective method for correcting excessive background\textsuperscript{272}. They suggested that on this basis, OSC is likely to find widespread application in pattern recognition for high-information-density data sets such as metabolomics.

Once the data is pre-processed classification of the biomarkers is made using linear methods, which use loading values such as PLS (partial least squares regression) or OPLS (orthogonal projections to latent structures). A multivariate classification method, PLS-DA, based on PLS, the regression extension of PCA\textsuperscript{282} shows maximum separation between the identified
subgroups in the data and once calculated and validated can be used to predict into which class metabolites fit. Whilst it has been reported that PLS-DA overfits the data and stringent validation is necessary\textsuperscript{282}, it has been successfully used in numerous studies, including identifying markers for coronary heart disease in humans using the NMR platform\textsuperscript{2242} and comparing the changes in the metabolome of urine in rats, mice and humans with type 2 diabetes\textsuperscript{283}. Orthogonal projections to latent structures, whereby OSC is performed prior to PLS, has the benefit of reduced unwanted variation. The results indicate that the OPLS-DA has a good capability for the classification of the prediabetic state and/or diabetic status\textsuperscript{284} in ddY mice. Based on the above, the best method(s) to be adopted to study metabolomics in healthy and obese cats should include supervised multivariate analysis along with pattern recognition via RLA, PCA, and volcano plots along with cross validation.

**Metabolomic studies in obesity and diabetes**

A very prolific area of research in metabolomics is the field of obesity\textsuperscript{285-287}, insulin resistance\textsuperscript{284,288-290} and type 2 diabetes\textsuperscript{255,291-293}. In diabetes and obesity research, the non-targeted approach has provided the opportunity to assess large human populations or investigate a range of different tissues in animal studies both rapidly and cheaply. Numerous metabolites have been identified as novel biomarkers for insulin resistance and type 2 diabetes in humans.

Several studies on the metabolomics of type 2 diabetes have been performed in vivo using mice. The db/db mouse has a single-gene autosomal recessive defect in the leptin receptor gene\textsuperscript{283}. The db/db mouse produces clinical signs of leptin resistance, hyperphagia, obesity, and subsequent insulin resistance. The obese Zucker (fa/fa) rat also has a single-gene autosomal recessive mutation in the leptin receptor, causing clinical signs of leptin resistance,
obesity, hyperlipidaemia, hyperinsulinaemia, and, post-6 weeks of age, fasting hyperglycaemia and type 2 diabetes. Metabolomic studies were performed on samples from diabetic mice (ddY-H) and normal mice (ddY-L) were globally separated by ultra-performance liquid chromatography (UPLC) and detected by electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS). Biomarker candidates related to diabetes mellitus were extracted from a multivariate statistical analysis. N-acetyl-L-leucine, an endogenous compound, was found in all biological specimens (plasma, hair, liver and kidney) and was therefore identified as a potential candidate biomarker related to diabetes. In another study, NMR was used to detect metabolomics in urine samples highlighted at least 24 distinct pathways that distinguish diabetic and control mice. The pathways most affected were amino acid degradation (threonine, valine, lysine, leucine, isoleucine, glycine, tyrosine, tryptophan), amino group metabolism (creatinine synthesis and degradation) and the urea cycle (glutamate metabolism and the ornithine pathway). A set of mouse liver samples to study the development and progression of insulin resistance. Using both GC–MS and GC × GC–MS, a total of 170 and 691 peaks, respectively, were quantified and preserved and several extra candidate biomarkers for insulin resistance were found.

Multivariate statistical analyses of proton NMR data from human, Zucker rat, and db mouse urine demonstrate metabolic similarities among the three species involving glucose metabolism, the Krebs cycle and the nucleotide and methylamine metabolism. It was speculated that this could provide markers not only for detecting diabetes in humans, but also for following its progression, although another study suggested that this was overclaiming.

Numerous studies have identified the metabolite profiles of diabetes in humans.
Investigations on whether metabolite profiles could predict the development of diabetes have been reported\(^{296}\). Five branched-chain and aromatic amino acids had highly significant associations with future diabetes: isoleucine, leucine, valine, tyrosine and phenylalanine. A combination of three amino acids predicted future diabetes. These findings underscore the potential key role of amino acid metabolism early in the pathogenesis of diabetes and suggest that amino acid profiles could aid in diabetes risk assessment\(^{296}\). Numerous other studies have supported the finding of amino acids as potential markers of type 2 diabetes in humans\(^{297-300}\). Increased bone resorption and consequent possible risk of fractures has been reported in diabetic patients by measuring increased urinary excretion of hydroxyproline and that this is related to alterations in renal function\(^{301}\). No such evidence has been reported in cats and to the authors’ knowledge no data is available on increases in hydroxyproline in the urine of diabetic cats.

Metabolic profiling using GC-MS via multivariate methods was applied to a small pilot study of samples from patients with impaired fasting glucose\(^{263}\). The results showed that the metabolomic patterns of the two groups were different and the linear combination of eleven discriminators: lactate, 2-ketoisocaproic acid, alanine, alpha-hydroxyisobutyric acid, urea, phosphoric acid, alpha-glycerophosphoric acid, palmitic acid, stearic acid, 1-monopalmitin and 1-monostearin, may be considered as the potential biomarkers of impaired fasting glucose. Plasma and urine of 51 non-diabetic humans fasted overnight was investigated aiming to separate subjects with IGT from controls, to investigate prediabetes\(^{302}\) and identified prediabetes associated alterations in fatty acid-, tryptophan-, uric acid-, bile acid-, and lysophosphatidylcholine- metabolism, as well as the TCA cycle. Studies of 40 individuals with self-reported diabetes and 60 controls (males, over 54 years of age) reported perturbations of metabolic pathways linked to kidney dysfunction (3-indoxyl sulfate), lipid
metabolism (glycerophospholipids, free fatty acids), and interaction with the gut microflora (bile acids)\textsuperscript{303}. The study suggests that metabolic markers hold the potential to detect diabetes-related sub-clinical conditions in the general population. Studies of 263 non-obese Asian-Indian and Chinese men demonstrated that alterations in amino acid homeostasis, but not inflammatory markers or free fatty acids, are associated with insulin resistance in lean humans\textsuperscript{297}. Numerous other studies support aberrations in lipid metabolism in prediabetes and type 2 diabetes in humans\textsuperscript{263,287,304,305}.

The biomarker alpha-hydroxybutyrate (alpha-HB) has been studied using ultra high performance liquid chromatography-mass spectrometry and GC-MS\textsuperscript{306}. A non-targeted (global) approach was conducted in 399 non-diabetic subjects, representing a broad spectrum of insulin sensitivity and glucose tolerance (based on the hyperinsulinaemic euglycaemic clamp and oral glucose tolerance testing, respectively). Alpha–HB was identified as a biomarker identifying clamp-derived insulin resistance in subjects with normal glucose tolerance and correlated with IFG and IGT independently of, and in addition to, insulin resistance. Importantly, these associations were independent of sex, age, and BMI. Thus, together with other biomarkers, alpha–HB may provide a diagnostic tool to identify insulin resistance and/or IGT earlier than currently used clinical tests\textsuperscript{306}.

Oral glucose tolerance tests have been used in humans to identify glucose intolerance and prediabetes\textsuperscript{10}. Numerous studies using this technique have been performed to better understand the effect of simulated hyperglycaemia in both rats and man. For example, the oral glucose tolerance test effect in healthy and diabetic rats and the metabolic changes in tissues of rats fed with high-fructose diet was studied using mass spectrometry based metabolomics\textsuperscript{278}. Several differentiating metabolites were identified involving amino acid
biosynthesis, polyunsaturated fatty acids, phospholipids and purine metabolism.

In humans, a study identified the metabolic pattern in plasma during an oGTT in healthy subjects\textsuperscript{266}. Free fatty acids, acylcarnitines, bile acids, and lysophosphatidylcholines were detected as the most discriminating oGTT biomarkers. Fatty acid levels were also found to be strongly decreased during the oGTT in humans using chromatography/mass spectrometry-based metabolomics\textsuperscript{307}.

Glyoxylic acid (glyoxylate) was found to be increased in humans at risk of developing diabetes mellitus when metabolite profiling was performed on a total of 177 healthy subjects, 121 prediabetic subjects, 30 diabetic subjects diagnosed by fasting blood glucose concentrations and 28 diabetic subjects diagnosed by impaired glucose tolerance. A new metabolic footprint, consisting of glyoxylate and a panel of metabolites known to be associated with diabetes and its complications, was found to be significantly altered in plasma samples of non-fasted subjects\textsuperscript{228}. When prediabetic subjects were compared to controls, glyoxylate was significantly increased in subjects primarily classified by IFG as well as in those primarily classified by IGT. The relationship between glyoxylic acid and type 2 diabetes has been supported by other studies\textsuperscript{285,308}.

The application of metabolomics in veterinary medicine is still in the early stages but the potential is vast\textsuperscript{224}. A recent report of the metabolomic changes associated with weight gain in dogs, suggest there are increases in pyruvate and mannose, some long chain fatty acids and lysophospholipids, homocysteine, cysteine, cystine and decreases in citrate α-ketoglutarate and anhydrogluticol\textsuperscript{309}. Additionally, the effect of dietary macronutrients on the feline plasma metabolome was studied using LC and GC-MS\textsuperscript{260}. It was reported that cats fed high protein
(50% ME), high fat (50% ME) and high carbohydrate (50%) diets had distinct clusters using principal component analysis. The profile of cats fed a high protein diet had a decreased nucleotide catabolism and increased amino acid metabolism, those fed a high fat diet showed increased lipid metabolism, but few changes were seen in cats fed the high carbohydrate diet when compared to cats fed the control diet.

Given the similarities between the pathophysiology of type 2 diabetes in humans and diabetes in cats, it is likely that metabolomic derangements in early disease may also be the same. To date there have been no studies published on the use of metabolomics to help diagnose prediabetes (insulin resistance) in cats though markers of obesity in Burmese cats were studied briefly\textsuperscript{65}.

The aim of our study is to discover if metabolomics could fulfil our goal of establishing effective clinical screening tests for prediabetes in cats.
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Chapter 2: Introduction

The prevalence of diabetes in cats varies from 0.3% to 2%, depending on the population studied; Burmese cats originating from Europe and Australasia are 4 times more likely to develop the disease\textsuperscript{1,2}, as are Russian Blue, Maine Coon and Siamese from the USA\textsuperscript{3}. In humans, the term prediabetes is used to classify a metabolic stage intermediate between normal glucose homeostasis and diabetes; these patients have impaired glucose tolerance and/or impaired fasting glucose\textsuperscript{4,5}. Either impaired glucose tolerance or impaired fasting glucose is identified in the previous 5 years in more than 60 % of humans who develop diabetes\textsuperscript{6}. In fact, it is reported that 33% of people with impaired glucose tolerance develop diabetes over a two year period\textsuperscript{7}. If prediabetes is identified, management through a low carbohydrate diet and weight loss regime can be implemented to delay or even prevent the onset of overt diabetes.

In cats, as in humans, impaired fasting glucose and impaired glucose tolerance can progress to clinical diabetes. Within 9 months of testing, cats in diabetic remission with moderate impaired fasting glucose or glucose intolerance developed diabetes (75% and 38% respectively)\textsuperscript{8}. Impaired fasting glucose is rarely diagnosed in cats unlike in humans, where 12 to 26% have impaired fasting glucose\textsuperscript{9} in USA, Europe and Australia, increasing to 39% by the age of 65 years\textsuperscript{10}. Mild increases in blood glucose in cats are often classified as stress hyperglycaemia associated with travel, a visit to a clinic or with illness\textsuperscript{6,11}. The identification prediabetic cats and cats with subclinical diabetes is likely to be as important as in humans to help prevent progression to clinical disease. An objective screening test similar to that used in humans would aid in identification of high risk cats, so that control measures can be implemented to help decrease the risk of clinical diabetes.
The aims of this thesis were to design and validate screening tests for prediabetes in senior cats, and to identify metabolomic changes in two groups of cats at high risk of developing diabetes - obese and Burmese.

A recent study in dogs reported a spurious effect on evaluation of glucose tolerance when glucose was dosed on a bodyweight basis in obese dogs, resulting in higher peak glucose concentrations and higher area under the glucose curve values, incorrectly implying impaired glucose tolerance\(^{12}\). For the analysis of glucose tolerance, this was resolved by adjusting the measured glucose concentrations, based on peak glucose concentration. The effect of obesity on the dosage of glucose used in a glucose tolerance test has not previously been investigated in cats. The primary objective of the study in Chapter 4 (“Dosing obese cats based on bodyweight spuriously affects some measures of glucose tolerance”; published) was to investigate whether dosing glucose by body weight results in spurious effects on measures of glucose tolerance in obese cats, because volume of distribution does not increase linearly with body weight. The aim was to retrospectively analyse glucose concentration data from glucose tolerance and insulin sensitivity tests in cats before and after they were fed *ad libitum* for 9 to 12 months to promote weight gain, to determine the adjustment required to compensate for the ‘dosage’ effects of obesity in overweight and obese cats.

The risk of developing type 2 diabetes in cats is highest after 8 years of age\(^{13}\). Humans with fasting and 2-h blood glucose concentrations that are above normal following a glucose tolerance test but below diabetic are classed as having impaired fasting glucose or impaired glucose tolerance respectively; they are considered prediabetic\(^{14}\). However, such data are not available for senior cats and a lack of standardization of these tests was evident. Additionally, these tests were found to not be convenient to use in a clinical environment. The ‘gold standard’ intravenous glucose tolerance test requires multiple samples and analysis is not practical in private practice because of the complex calculations required to generate the
necessary statistics, such as glucose half-life, glucose clearance time, and area under the
curve\textsuperscript{15}. The aim of Chapter 5 (“Diagnosis of prediabetes in cats: glucose concentration
cutpoints for impaired fasting glucose and impaired glucose tolerance”; published) was to
establish the methodology and cutpoints for fasting and 2-h blood glucose concentrations in
healthy client-owned senior cats ($\geq 8$ years of age) using ear/ paw samples and a portable
blood glucose meter calibrated for feline blood.

Casual blood glucose, defined as blood glucose measured unrelated to time of eating or type
of food, is used as a screening test to diagnose diabetes in humans\textsuperscript{4}. No standardized
methodology and cutpoints for normal have been established for cats. The aim of Chapter 6
(“Cutpoints for screening blood glucose concentrations in healthy senior cats”; in press) was
to standardize the methodology for a screening test similar to that used in humans, and
develop reference intervals which would aid identification of high risk cats, that is, cats with
impaired fasting glucose or subclinical diabetes, so that interventions can be implemented to
decrease the risk of progression to clinical diabetes.

Metabolomics is a new method using liquid or gas chromatography together with mass
spectroscopy to measure a comprehensive set of metabolites in a given body fluid or tissue
extract\textsuperscript{16}. In the progression of disease alterations in the metabolic profile often present much
earlier than clinical signs. Therefore, metabolomics can potentially be used as a sensitive,
early indicator of a disease process. There is scant information on metabolite changes with
obesity in pets, despite obesity being similar in dogs and cats to humans\textsuperscript{17-20}. The aim of
Chapter 7 (“Metabolomic differences between lean and obese neutered senior cats and
associations with glucose tolerance”) was to identify the metabolic profile in spontaneous
obesity in senior neutered cats, and to determine associations with measures of glucose
metabolism and diabetes.
Burmese cats from the UK, Sweden, Australia and New Zealand are now almost four times more likely to develop type 2 diabetes mellitus than other cats.\textsuperscript{1,21,22,23} Reports show that 28% of Australian Burmese cats have altered lipid metabolism as evidenced by an exaggerated postprandial triglyceride response after an oral fat tolerance test\textsuperscript{24,25} which possibly could contribute to the increased risk of diabetes. Chapter 8 ("Metabolite differences between healthy senior Burmese and non-Burmese cats, and associations between metabolites and measures of glucose metabolism") investigated metabolite differences between healthy Burmese and non-Burmese cats, after adjusting for age, sex, and body condition score. Again, like obese cats (Chapter 7), associations between identified metabolites and measures of glucose metabolism and diabetes were examined.

The overall aim of the thesis was to establish a screening test that is easy to perform for prediabetes in senior cats to be used in a clinical setting, and to improve the understanding of the metabolic differences in cats at high risk of developing diabetes, namely obese and Burmese senior cats, in the hope of helping prevent clinical disease in the future.
References


Chapter 3: Study design diagrams

The following pages are diagrammatic summary of the major experiments of this thesis.

Experiment 1

Aims

- To investigate changes induced by obesity in absolute glucose concentrations measured at various times after a bolus dose of intravenous glucose
- To investigate the effect of these changes on the diagnosis of glucose intolerance based on 2-h glucose concentrations
- Such information will be important to underpin the development of reference values for a simple clinical test for glucose intolerance in cats that requires only a fasting and a 2-h blood sample.

Animals

Data analysed retrospectively from 16 cats (aged 1-5 years, BCS 1-5/5). All cats were clinically healthy. All cats were acclimatized to their environment and fed the same food.

Methods

- All cats were anaesthetized and jugular catheters inserted at least 24 h prior to tests and food was withheld for 12 h.
- Glucose tolerance tests were performed at an IV glucose dosage of 0.3g/ kg and 1.0 g/kg bodyweight before and after adlib feeding which led to weight gain.

Relevant research chapters

Chapter 4- Dosing obese cats based on body weight spuriously affects some measures of glucose tolerance
Experiment 2

**Aims**

- To design a simplified intravenous glucose tolerance test to use in a clinic environment using paw/pinna samples and a portable glucose meter
- To determine precision and accuracy of a portable glucose meter
- To establish cutpoints for fasting and 2-h blood glucose concentrations in healthy cats 8 years and older
- To apply the adjustment equations from experiment 2 to 2-h blood glucose concentrations in overweight and obese cats
- To compare glucose tolerance tests of healthy neutered Burmese cats to healthy neutered domestic cats of the same age group and matched body condition score

**Animals**

Healthy client-owned cats 8 years and older (n=78) were classified as non-Burmese (n=59) or Burmese (n=19). Body condition scores (out of 9) were 4 or 5 (27 cats), 6 or 7 (31 cats), 8 or 9 (20 cats).

**Methods**

Cats were fasted for 18-20 hours and a cephalic catheter inserted. Three hours later, a simplified IVGTT was performed using 0.5 g/kg body weight intravenous glucose (50% dextrose solution) administered through the cephalic catheter. Blood glucose concentration was measured using a portable glucose meter (Abbott AlphaTRAK) from the ear or paw immediately before glucose injection (time 0) and after glucose injection at 2 min and 2 h.

**Relevant research chapters**

*Chapter 5- Diagnosis of prediabetes in cats: glucose concentration cut points for impaired fasting glucose and impaired glucose tolerance*
Experiment 3

Aims

- To design the methodology for a blood glucose screening test to be used in a clinic environment
- To determine cutpoints in cats for screening blood glucose concentration measured from paw or ear samples with a portable glucose meter
- To compare these values with those obtained in the traditional way
- To determine whether there are associations between screening blood glucose and weight, body condition score, stress score, diet (amount of carbohydrate eaten in previous 6, 12 and 24 h) or breed (Burmese versus non-Burmese)

Animals

120 healthy neutered client-owned cats 8 years and over of varying weights and body condition scores

Methods

On entering the consultation room, blood glucose concentration was measured using a portable glucose meter in blood obtained from the paw or ear via a lancing device. A history and clinical examination was performed, followed by collection of a jugular blood sample. Blood glucose concentration was measured with the portable meter and at a reference laboratory to determine meter accuracy. Data on signalment, health history, type of food, amount and timing of eating in the previous 24 h was obtained. Behaviours associated with stress at the time of the initial blood collection were scored.

Relevant research chapters

Chapter 6- Cutpoints for screening blood glucose concentrations in healthy senior cats
Experiment 4

Aim

- To establish what metabolites are changed with obesity in elderly cats
- To establish if any individual metabolites are associated with measures of glucose metabolism (screening/fasting and 2-hr blood glucose following a glucose tolerance test) and diabetes related parameters (insulin, G:I ratio, leptin, triglycerides, adiponectin, leptin:adiponectin ratio, fPLi and MCP-1

Animals

Cats volunteered by their owner from experiment 2 and 3 and of appropriate demeanour

Samples from 39 cats were used in this study of metabolomics: 21 lean (BCS4-5/9) non-Burmese (13 domestic and 8 other breeds) and 18 obese (BCS 8-9/9) non-Burmese (15 domestic and 3 other breeds).

Methods

- A jugular venous blood sample was collected from cats fasted for the glucose tolerance test in experiment 2
- Serum and plasma was separated into aliquots for hormonal, enzymatic and metabolomic (GC-MS) analysis and stored at -80°C until analysis

Relevant research chapter

Chapter 7- Metabolomic differences between lean and obese neutered senior cats and associations with glucose tolerance
Experiment 5

Aim

- To compare the metabolites between healthy Burmese and non-Burmese cats 8 years and older
- To compare these significant metabolomic variables with fasted insulin, G:I ratio, triglycerides, leptin, adiponectin, leptin:adiponectin ratio, fPLi and MCP-1 and with measures of glucose intolerance (fasting blood glucose and 2-h blood glucose following a glucose tolerance test) in senior Burmese and non-Burmese cats.

Animals

Cats used in this study were from experiment 2 and 3, volunteered by their owner and of appropriate demeanour.
Samples from cats (n=49) classified as non-Burmese (n=30) or Burmese (n=19) were used in this study. Body condition scores for non-Burmese were 12 lean (BCS 4-5), 5 overweight (BCS 6-7) and 13 obese (BCS 8-9) (mean 6.1, range 4-9) and for Burmese were 6 lean, 11 overweight and 2 obese (mean 6.1, range 4-8).

Methods

- A jugular venous blood sample was collected from cats fasted for the glucose tolerance test in experiment 2
- EDTA plasma and serum were separated into aliquots for hormonal and metabolomic analysis and stored at -80 degrees until analysis until analysis

Relevant research chapter

Chapter 8- Metabolite differences between healthy senior Burmese and non-Burmese cats, and associations between metabolites and measures of glucose metabolism
Chapter 4: Dosing obese cats based on body weight spuriously affects some measures of glucose tolerance

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Abstract

The primary objective was to investigate whether dosing glucose by body weight results in spurious effects on measures of glucose tolerance in obese cats, because volume of distribution does not increase linearly with body weight. Healthy research cats (n = 16; 6 castrated males, 10 spayed females) were used. A retrospective study was performed using glucose concentration data from glucose tolerance and insulin sensitivity tests before and after cats were fed *ad libitum* for 9 to 12 mo to promote weight gain. The higher dose of glucose (0.5 versus 0.3 g/kg body weight) in the glucose tolerance tests increased 2-min glucose concentrations (*P* <0.001), and there was a positive correlation between 2-min and 2-h glucose (r = 0.65, *P* = 0.006). Two-min (*P* = 0.016 and 0.019, respectively), and 2-h (*P* = 0.057 and 0.003, respectively) glucose concentrations and glucose half-life (T ½) (*P* = 0.034 and <0.001 respectively) were positively associated with body weight and body condition score. Glucose dose should be decreased by 0.05 g for every kg above ideal body weight. Alternatively, for every unit of BCS above 5 on a 9-point scale, observed 2-h glucose concentration should be adjusted down by 0.1 mmol/L. Dosing glucose based on body weight spuriously increases glucose concentrations at 2 h in obese cats and could lead to cats being incorrectly classified as having impaired glucose tolerance. This has important implications for clinical studies assessing the effect of interventions on glucose tolerance when lean and obese cats are compared.
Introduction

The IVGTT was introduced in human medicine in 1923 to assess glucose homestasis\(^1\) and measures an individual’s ability to clear glucose from circulation after a bolus dose of glucose. People with impaired glucose tolerance are considered prediabetic and at high risk of developing type 2 diabetes\(^2\)\(^-\)\(^4\). Measures of glucose tolerance include T \(\frac{1}{2}\) and rate of glucose clearance, but these require multiple blood samples and complex mathematical calculations. In clinical practice, blood glucose concentration two hours after a standardized oral glucose dose (75g/adult person) is currently used to identify humans with impaired glucose tolerance\(^5\)\(^,\)\(^6\).

Approximately 1 in 200-400 domestic cats and 1 in 50 Burmese cats of European origin develop diabetes analogous to human type 2 diabetes\(^6\)\(^-\)\(^10\). As in humans, if diagnosed in the prediabetic state, clinical disease can likely be averted or minimized through diet and weight loss\(^11\). However, tests for prediabetes are not well characterised or commonly utilized in clinical veterinary practice, and cats are usually not diagnosed until overt diabetes is evident\(^8\).

A number of criteria for classification of glucose tolerance in cats have been published\(^9\)\(^,\)\(^12\)\(^,\)\(^13\). The “gold standard” test is tedious, requires multiple blood samples, and interpretation can be difficult because of the complex calculations required, such as glucose T \(\frac{1}{2}\), rate of glucose clearance, and area under the curve\(^14\)\(^,\)\(^15\). For many cat owners, the procedure is unacceptably expensive and invasive. A routine screening test equivalent to the test in human medicine, based on absolute glucose concentrations and requiring two blood samples, was reported in 1998 but has not been widely implemented for cats\(^14\). Although there are inherent problems associated with interpreting absolute glucose values\(^16\), these measures form the basis of a satisfactory primary screening test for prediabetes in humans\(^3\) and could be similarly used for cats.
In humans, oral administration of glucose has replaced intravenous administration, due to its ease and practicality\(^3\). Oral glucose administration has been reported in cats and, as in humans, results in greater insulin stimulation through an incretin effect\(^{17}\). However, cats are very susceptible to stress hyperglycaemia and may be stressed by oral administration which would confound the assessment of glucose tolerance\(^{18}\). Stress, particularly if it results in struggling, can increase blood glucose concentrations by as much as 10 mmol/L and take 3 h to resolve\(^{18}\), and therefore needs to be avoided. Placing a cephalic catheter for glucose injection 3 h before the test start likely assists in preventing struggling and stress-induced hyperglycaemia during the test.

A recent study in dogs reported a spurious effect on evaluation of glucose tolerance when glucose was dosed on a body weight basis in obese dogs, resulting in higher peak glucose concentrations and higher area under the glucose curve, incorrectly implying impaired glucose tolerance\(^{19}\). For the analysis of glucose tolerance, this was resolved by adjusting the measured glucose concentrations, based on peak glucose concentration. The observation that peak glucose concentration was higher in obese dogs compared to lean dogs when dosing glucose based on body weight is not surprising. For a substance injected intravenously, the volume of distribution (and circulating blood volume), expressed as a percentage of body weight, is reduced in obese individuals, because it does not increase linearly with increasing fat mass\(^{19-21}\). Therefore, when administering glucose at a dose based on body weight, obese cats are potentially overdosed in comparison with cats in ideal body condition, which could lead to a false assessment of impaired glucose tolerance. No studies investigating clearance of anaesthetic agents or sedatives or allometric scaling in cats have been identified\(^{22}\).

The purpose of this study was to investigate effects of changes induced by obesity on absolute glucose concentrations measured at various times after a bolus dose of intravenous glucose. We then determined the effect of these obesity-induced changes on assessment of
glucose homeostasis, in particular, diagnosis of glucose intolerance, and investigated whether adjustment for glucose dose was indicated in obese cats. Such information is important for the development of reference values for a simple clinical test for glucose intolerance in cats that requires only a fasting and a 2-h blood sample.
Materials and Methods

A retrospective study was conducted using data from a previous study that investigated the effect of obesity on various glucose variables. That study was approved by the Animal Experimentation and Ethics Committee of the University of Queensland. In the current study, we examined the effects of body weight and body condition on variables including peak and 2-min glucose after glucose administration, fasting glucose, glucose dose (0.3 and 0.5 g/kg), and measures of glucose tolerance such as T½ and 2-h glucose concentration. We utilized blood glucose concentration data from glucose tolerance tests and the first 2 min from insulin sensitivity test previously performed at our laboratory in 16 cats before and after weight gain. In the original study, 16 (10 spayed females, 6 castrated males) clinically healthy research cats between 1 and 5 yr of age (most in ideal body condition) underwent glucose tolerance and insulin sensitivity tests to provide baseline data prior to weight gain. The cats were offered a combination of 2 commercially available extruded foods, with metabolizable energy consisting of 33% protein, 22.3% fat, 30.2% carbohydrate and 40% protein, 26.6% fat and 17.2% carbohydrate, respectively. The tests were repeated after a weight gain period, when cats were fed ad libitum for an average (± standard deviation) of 10.5 (± 1.1 mo) (range, 9 to 12 mo). Based on dual energy x-ray absorptiometry, after the weight gain period, all cats had more than 30% body fat (range 34.2 – 48.7%). Body condition score was originally measured on a 5-point scale. For the current study, these scores were converted to a 9-point scoring system, where scores of 1, 2, 3, 4, and 5 on the 5-point scale were converted to 1, 3, 5, 7, and 9, respectively, on the 9-point scale to allow better comparison with other data sets. All body condition scores were measured by the same person.

In the original study, glucose tolerance and insulin sensitivity tests were performed on separate days. For the glucose tolerance test, glucose was administered at 0.5 g/kg as a
bolus dose over 30 s, via a jugular vein catheter. Blood samples were collected before glucose administration (four samples at -15, -10, -5, and -1 min) and after (at 2, 5, 10, 15, 30, 45, 60, 90, and 120 min). For the insulin sensitivity test, glucose was administered at 0.3 g/kg as a bolus dose over 30 s, via a jugular vein catheter and insulin was injected 20 min later. Glucose concentration data before and 2 min after glucose injection in the insulin sensitivity test was used to compare the effect of glucose dose on 2-min blood glucose concentration after injection of 0.3 g/kg glucose and 0.5 g/kg from glucose tolerance test. Data from blood samples collected after insulin administration could not be used due to effects of insulin on glucose concentrations, precluding the calculation of T ½ and time to return to baseline for the 0.3g/kg dose rate. In both tests, the timer was started halfway through infusion. Glucose was measured in plasma using an automated glucose analyser which had a precision for replicate analyses of < 2% (YSI 2300 Stat Plus; Yellow Springs Instrument Co.)

Statistical analyses

The statistical analyses assessed a) the effects of weight gain on 2-min and peak blood glucose concentration by comparisons before and after the 9 to 12 mo ad libitum feeding period, b) the effects of glucose dose (0.3 g/kg versus 0.5 g/kg) on 2-min glucose, and c) selected associations between fasting blood glucose concentration, 2-min and peak blood glucose concentrations, body weight, body condition score, and key measures of glucose tolerance, namely T ½ and 2-h blood glucose concentration. Linear regression and correlation coefficients were used. Statistical analyses were performed using specialist software (Stata version 12, StataCorp, College Station, Texas, USA). T ½ for glucose was estimated for each cat using glucose values from 15 to 90 min. Between these time points, the glucose disappearance curve is most rectilinear. \( \log_e (\text{glucose}) \) was regressed on time (in min) and T_{1/2} was calculated using the resulting \( \beta \)-coefficient (ie. the slope coefficient) as follows: T_{1/2}
= \log_e(2)/|\beta\text{-coefficient}|. This was equivalent to fitting the model: \( G_t = G_0 \times e^{(-k\cdot t)} \), where \( G_t \) is the serum glucose concentration at time \( t \), and \( G_0 \) is the initial serum glucose concentration based on extrapolation from the period from 15 to 90 min to time 0\textsuperscript{27}.

For most analyses, data from before and after the \textit{ad libitum} feeding period were pooled for linear regression analyses. Thus the ‘cat-period’ was the unit of analysis where each cat provided 2 'cat-periods', 1 before and 1 after it was fed \textit{ad libitum}. Cat was fitted as a random effect (to account for clustering of cat-period within cat), using maximum likelihood estimation. R-square values overall and within cat were estimated using models fitted using the generalized least squares estimator. Additionally, confounding by sex of relationships assessed using linear regression was explored by comparing regression coefficients after refitting the models with sex included as a covariate; interactions with cat sex were also assessed in subsequent models. Regression coefficients changed minimally after adjustment for sex, and P-values for all interaction terms were above 0.26, so results of these additional analyses were not reported. For correlation coefficients, 95% confidence intervals were calculated using Fisher's transformation.

Following an intravenous injection of glucose at time 0, the direct causal effect of 2-min glucose concentration on 2-h glucose concentration was estimated by regressing 2-h glucose concentration on 2-min glucose concentration with BCS fitted as a continuous covariate. The rationale for this analysis was that 2-h glucose concentration could be affected by 2-min glucose concentration via a 'direct' causal effect, if there was no (or insufficient) increase in rate of glucose clearance with higher 2-min glucose concentrations (Figure 4.1). Hence, higher 2-min glucose concentrations would result in higher 2-h glucose concentrations. This is supported by the observation that T \( \frac{1}{2} \) of glucose (a measure of glucose clearance) was not
closely related to peak glucose concentration (Figure 4.2). The rationale for fitting BCS was that BCS may affect 2-min glucose concentration if glucose dose is calculated on a body weight basis. This occurs because the volume of distribution does not increase linearly with increases in body weight due to accumulation of body fat. There may also be effects of obesity on 2-h glucose concentration mediated other than through higher 2-min glucose concentration. These effects could include increases in insulin resistance with obesity, which in turn, could increase 2-h glucose concentration. If so, BCS would confound the observed association between 2-min glucose concentration and 2-h glucose concentration, when glucose is dosed on a body weight basis. Associations between fasting blood glucose and each of 2-min and 2-h blood glucose concentrations were also assessed using linear regression. Because we postulated a priori that these relationships involving fasting blood glucose may have differed between before and after the 9 to 12 mo ad libitum feeding period, separate linear regression models were fitted for each period. Variances of 2-h glucose were compared between before and after the ad libitum feeding period using Levene’s test for the equality of variances between groups; this did not account for pairing of results within cat. Reference intervals for 2-h glucose concentration were calculated using published methods that were incorporated into a spreadsheet (Microsoft Excel spreadsheet, Reference Interval Draft Version, Copyright 2005, University of Cincinnati). Data were transformed to approximate a normal distribution using the Box-Cox transformation, and outliers identified for exclusion from subsequent calculations. Associated 90% confidence intervals for the upper 95% limits of the reference intervals for 2-h glucose concentrations before and after the ad libitum feeding period were estimated using bootstrapping with 1000 replications. Only cats with body condition scores of 8 or 9 (n = 10) were used to calculate the reference interval after the ad libitum feeding period.
Results

After the *ad libitum* feeding period, the average proportional body weight increase was 44% compared to the initial body weight (before weight gain mean ± SD of 4.4 ± 0.8 kg to after weight gain 6.3 ± 1.3 kg). Most cats were initially in ideal body condition (BCS 5 n=14; BCS 3 n=1; BCS 7 n=1). Body condition scores on the 9-point scale increased from a mean ± SD of 5.0 ± 0.7 to 8.1 ± 1.1. After weight gain, 10 of the 16 cats were classed as obese (BCS 8 or 9) and 6 as overweight (BCS 6 or 7). All results were based on a glucose dose of 0.5 g/kg body weight IV unless otherwise stated.

*Effects of weight gain on 2-min and peak blood glucose concentrations*

When glucose dose was based on body weight (ie. 0.5 g/kg body weight), both 2-min and peak blood glucose concentrations were significantly increased by weight gain (ie. from before to after the weight gain period; *P* ≤ 0.001; Figure 4.3). Peak blood glucose concentrations occurred at 2 min for 14 of the 16 cats and at 5 min in 2 cats, both before and after the *ad libitum* feeding period. Peak glucose concentration was closely correlated with 2-min glucose both before and after the *ad libitum* feeding period (*r* = 0.96; 95% CI 0.88 to 0.99; *P* < 0.001 and *r* = 0.98; 95% CI 0.95 to 1.00; *P* < 0.001, respectively).

Two-min glucose increased with each of body weight and body condition score (Table 4.1). Body weight explained 15% of the total variation, and 26% of the within-cat variation for 2-min glucose concentrations. Body condition score explained 15% and 18% of the total and within-cat variations, respectively, for 2-min glucose concentration.

* Associations between peak and 2-min blood glucose concentrations and measures of glucose tolerance
Before the *ad libitum* feeding period, both peak and 2-min glucose concentrations were positively correlated with 2-h blood glucose concentration (peak: \( r = 0.61; \) 95% CI 0.17 to 0.85; \( P = 0.012 \); 2-min: \( r = 0.65; \) 95% CI 0.23 to 0.87; \( P = 0.006 \)) (Figure 4.4). Using pooled data from before and after the *ad libitum* feeding period, for every 1 mmol/L increase in 2-min glucose concentration, 2-h glucose concentration increased by 0.09 mmol/L (95% CI -0.01 to 0.18 mmol/L; \( P = 0.067 \)).

*Associations between body weight and BCS and measures of glucose tolerance*

Using pooled data from before and after the *ad libitum* feeding period, 2-h glucose was associated with body weight (estimated increase for each extra kg body weight 0.33 mmol/L; 95% CI -0.01 to 0.67; \( P = 0.057 \)) and BCS (estimated increase for each extra unit of BCS 0.32 mmol/L; 95% CI 0.11 to 0.53; \( P = 0.003 \)) (Table 4.1, Figure 4.5). Body weight and BCS explained 7% and 15%, respectively, of the total variation in 2-h glucose concentrations, and 30% and 38%, respectively, of the within-cat variation. The variance of 2-h glucose was significantly larger (\( P = 0.013 \)) after the *ad libitum* feeding period; standard deviations were 0.9 mmol/L and 1.6 mmol/L before and after the *ad libitum* feeding period, respectively.

The upper limit for the 95% reference interval for 2-h glucose concentration using cats before the *ad libitum* feeding period was 6.0 mmol/L (90% CI 5.1 to 6.6 mmol/L) and using cats after the *ad libitum* feeding period was 8.9 mmol/L (90% CI 7.1 to 9.3 mmol/L).

Increasing body weight and body condition score were associated with increasing T ½ (Table 1). Body weight and BCS explained 9% and 19%, respectively, of the total variation in T ½, and 27% and 45%, respectively, of the within-cat variation.

*Association between fasting glucose and each of 2-min and 2-h glucose concentrations*
Both 2-min and 2-h blood glucose concentrations were positively associated with fasting blood glucose concentrations both before and after the *ad libitum* feeding period (P ≤ 0.018).

**Effect of glucose dose rate (0.3 versus 0.5 g/kg) on 2-min blood glucose concentrations**

The dose rate of glucose (0.3 versus 0.5 g/kg body weight) affected 2-min glucose concentrations. 2-min glucose concentration increased from a mean ± SEM of 18.0 ± 0.8 mmol/L to 22.7 ± 1.3 mmol/L (P < 0.001) when glucose dose was increased from 0.3 to 0.5 g/kg body weight. 2-min glucose increased by 4.7 mmol/L (95% CI 2.1 to 7.2 mmol/L) when the dose increased from 0.3 to 0.5 g/kg, equating to an increase in 2-min glucose concentration of 23.3 mmol/L for every 1g/kg increase in glucose dose. Effects of dose on T ½ and 2-h blood glucose concentrations could not be calculated because insulin was administered 20 min after the 0.3 g/kg dose of glucose was given and prevented valid measurement of these variables.

**Accounting for effects of obesity on 2-h glucose concentrations when using glucose tolerance tests**

To account for the spurious effects of obesity on 2-h glucose concentrations when dosing glucose by body weight, a number of simple theoretical options were investigated. These options were designed to allow results from cats with body condition scores varying from lean to obese to all be interpreted using the upper limit of the reference interval for 2-h glucose concentration calculated for lean cats. Two approaches were investigated: adjustment of glucose dose and adjustment of measured 2-h glucose concentration.

**Adjustment of glucose dose:**
For every extra unit of BCS above 5 on a 9-point scale, the glucose dose of 0.5 g/kg glucose should be reduced by 0.05 g/kg. This was calculated by the following equations:

Adjusted glucose dose = Actual glucose dose - \( X \), where \( X = \frac{A}{B} \)

Where \( X \) = Adjustment of glucose dose; \( A \) = Increase in 2-min blood glucose concentration/unit BCS = 1.14 mmol/L (Table 4.1) and, \( B \) = Increase in 2-min glucose concentration/each 1g/kg increase in glucose dose = 23.3 mmol/L.

Similarly, if using body weight as a measure, for every kg increase in body weight above the cat’s ideal body weight (its body weight if it were BCS 5), the dose should be decreased by \( X = 0.064 \) g/kg, where

\( A \) = increase in 2-min blood glucose per kg increase in body weight=1.48 mmol/L (Table 4.1); \( B=23.3 \).

Adjustment of measured 2-h glucose concentration following a dose of 0.5 g/kg body weight.

This was calculated as follows:

Adjusted 2-h glucose concentration = actual 2-h glucose concentration – correction for obesity.

The correction of 2-h glucose concentration for obesity was calculated in 3 ways, based on associations between 2-h glucose concentrations and each of 2-min glucose concentrations, BCS, and body weight from this study. These 3 methods are for use in cats with a BCS greater than 5 (method a), or when body weight is greater than ideal (ie body weight is higher than if cat were BCS 5; method b), or cats with a 2-min glucose concentration > 17.8 mmol/L (method c).

Method a: Correction for obesity based on the effects of BCS on 2-min glucose concentration:

\[(\text{Cat’s actual BCS } - 5) \times 0.10 \text{ mmol/L.}\]
This calculation assumes that 5 is an ideal BCS on a 1 to 9 scale, and 0.10 mmol/L is the direct causal amount by which 2-h glucose concentration increases for each unit increase in BCS via an effect on 2-min blood glucose concentration. This was estimated by multiplying the amount by which the 2-min glucose concentration increased per unit increase in BCS (1.14 mmol/L; Table 4.1) by the estimated direct causal effect of 2-min glucose concentration on 2-h glucose concentration (0.09 mmol/L). For example, one study cat had a BCS of 9 and a 2-h blood glucose concentration of 6.2 mmol/L. As this is 0.2 mmol/L above the upper limit of the calculated 95% reference interval of 6.0 mmol/L, without adjustment, this cat would have been classified as having impaired glucose tolerance. Using this method of adjustment (0.01 mmol/L for each unit of BCS), this cat would have an adjusted 2-h glucose concentration of 5.8 mmol/L (Table 4.2) and so be classified as having normal glucose tolerance. In contrast, another study cat with a BCS of 7 and a 2-h glucose concentration of 7.4 mmol/L would have an adjusted 2-h glucose concentration of 7.2 mmol/L, and so would still be considered to have impaired glucose tolerance.

**Method b: Correction for obesity based on the effects of body weight on 2-min glucose concentration:**

(Cat’s actual weight – Cat’s ideal body weight) x 0.13 mmol/L,

In this calculation, the amount by which the 2-min glucose concentration increased per kg increase in body weight (1.48 mmol/L; Table 4.1) was multiplied by the estimated direct causal effect of 2-min glucose concentration on 2-h glucose concentration (0.09 mmol/L) resulting in an adjustment of 2-h blood glucose by 0.13 mmol/L per kg of weight increase from ideal body weight (Table 4.2).

**Method c: Correction for obesity based on the mean 2-min glucose concentration in cats of ideal BCS (BCS 5)**
(Cat’s actual 2-min glucose concentration – 17.8) x 0.09 in mmol/L.

In this calculation, 17.8 mmol/L was used as this was the mean 2-min glucose concentration for cats with ideal BCS (BCS 5; n=14 cats prior to the *ad libitum* feeding period), and 0.09 is the estimated direct causal effect of 2-min glucose concentration on 2-h glucose concentration. This method assumes that the 2-min glucose concentration for cats in ideal BCS (5) is always 17.8 mmol/L. For the cat in the previous example with a BCS of 9, the 2-min glucose concentration was 29.2 and the recalculated 2-h glucose concentration was 5.3 mmol/L. Similarly, for the cat with a BCS of 7, the 2-min glucose concentration was 21.7 mmol/L, and the recalculated value of 7 mmol/L would still be considered indicative of impaired glucose tolerance (Table 4.2).
Discussion

Glucose clearance after an intravenous glucose challenge, and therefore measured glucose concentrations, are influenced by complex mechanisms involving insulin and non-insulin dependent glucose uptake into tissues, as well as suppression of glucose output via gluconeogenesis in the liver\textsuperscript{31}. Obesity affects both receptor and post-receptor processes involved in glucose uptake into tissues, and inhibits suppression of hepatic gluconeogenesis. Obesity also adversely affects beta cell function and insulin secretion\textsuperscript{32}. These effects on glucose clearance are mediated by insulin resistance as well as other direct effects including inflammatory mediators associated with obesity\textsuperscript{16}.

Glucose tolerance is assessed by measures of glucose clearance such as glucose half-life ($T_{\frac{1}{2}}$), and absolute glucose concentrations at a given time after a glucose challenge, for example, 2 h\textsuperscript{33}. In cats, glucose is most commonly administered intravenously and dosed on a body weight basis\textsuperscript{12,16}. However, the volume of distribution of glucose does not increase linearly with increasing fat mass, potentially resulting in a relative glucose overdose in obese cats. Our study is the first study to examine whether dosing glucose on a per kg basis in an intravenous glucose tolerance test has a spurious effect on the results when testing overweight and obese cats. There were several important findings from this study that clinical researchers should be aware of when comparing glucose tolerance between lean and obese cats.

Firstly, glucose concentration measured at 2 min is positively associated with glucose dose. Secondly, the 2-min glucose concentration has a direct causal effect on the 2-h glucose concentration. Thirdly, both body condition score and body weight were positively associated with 2-min and peak glucose concentrations, 2-h glucose concentration, and $T_{\frac{1}{2}}$. These findings corroborate those of others\textsuperscript{12,16} and have implications for interpreting measures of
glucose tolerance, especially glucose concentrations at 2 h after glucose administration. There is the potential to erroneously classify an obese cat as having impaired glucose tolerance because of the effect of a standard glucose dose on peak or 2-min glucose, and subsequently on 2-h glucose concentration.

Peak (and 2-min) glucose concentration are a reflection of the volume of distribution, as no substantial clearance of glucose from the blood occurs in the short timeframe\textsuperscript{16,21}. The volume of distribution, that is the circulating blood volume, does not increase linearly with body weight in obesity\textsuperscript{34,35}. The important finding in our study is that there is relative overdosing of glucose in obese cats when glucose dosing is based on body weight. Further investigation would be required to examine if dosing on ideal body weight or using a fixed dose regardless of body weight, as used in human medicine, was advantageous in glucose tolerance tests in obese cats. Both of these methodologies incorrectly assume that volume of distribution (circulating blood volume) do not increase concomitant with increasing fat mass. However, blood volume indeed does increase with increasing fat mass, but at a proportionally lower rate compared to if the weight increase was muscle\textsuperscript{24}. The second limitation with dosing based on ideal body is the inherent imprecision of estimating ideal body weight in obese cats using morphometric or other measures available in clinical practice.

In human\textsuperscript{3,4,36} and veterinary\textsuperscript{8} medicine, fasting glucose concentration is also used as a measure of glucose homeostasis, and patients with increased concentrations are classified as either having impaired fasting glucose or diabetes, depending on the magnitude of the increase. Fasting glucose concentration is less sensitive for detecting abnormalities of glucose homeostasis than measurement of glucose concentrations after a glucose challenge, as occurs in the glucose tolerance test\textsuperscript{2,37,38}. $T\frac{1}{2}$ is based on 4 glucose measurements from 15-90 min and there was no evidence that $T\frac{1}{2}$ was affected substantially by peak values. Whether it is a
better tool to assess glucose tolerance status in obese cats, or is a less sensitive measure of glucose tolerance than absolute glucose concentrations, is unclear. However, it is technically more difficult to calculate and not suitable as a simple measure of glucose tolerance status for use in clinical research or veterinary practice. In clinical practice in humans, the 2-h glucose concentration has replaced \(T_{1/2}\) as a measure to identify individuals with impaired glucose tolerance and diabetes.

It is important to separate the dosage effect of obesity from the pathological effect of obesity on the 2-h glucose concentration. The pathological effect is associated with impaired glucose clearance from the circulating blood as a result of peripheral insulin resistance secondary to obesity and a number of other factors affecting glucose uptake into tissues and insulin secretion\(^\text{11}\). In our study, the combined dosage and pathological effects increased the 2-h glucose concentration by 0.32 mmol/L for every unit increase in body condition score. The calculated spurious effect of dosing on body weight was 0.10 mmol/L/unit increase in BCS, or approximately 30% of the overall increase in 2-h blood glucose induced by obesity. Our results showed a significant difference between the upper 95% reference interval for 2-h glucose concentrations in lean and obese cats (6 and 8.9 mmol/L), indicating that one reference limit for all cats was inappropriate. However, employing different reference limits for different body condition scores or body weights could cause confusion and be less practical. Based on our results, a simplified test for assessment of glucose tolerance—relying on a 2-h blood glucose measurement and a single reference range for normal cats—would need to account for the dosage effects of obesity. This could be achieved in overweight and obese cats by adjusting down either the dose of glucose administered, or the 2-h glucose value before interpretation with standardised reference limits.

Based on the associations found, we propose two ways to adjust the measured 2-h glucose value, to account for the dosage effects of obesity. Both methods attempt to account for the
effects of dose alone, and therefore involve the relationships between body weight or condition, 2-min, and 2-h glucose concentrations. The direct causal effect (spurious effect) of 2-min glucose concentration on 2-h glucose concentration was estimated by regressing 2-h glucose concentration on 2-min glucose concentration adjusted for BCS. Thus, our estimates of this relationship are independent of any decrease in insulin sensitivity in the obese cats. Adjustment using measured 2-min glucose is considered less ideal than using BCS because it relies on comparison with the calculated mean 2-min glucose concentration for lean cats, which may cause inaccuracies given that the standard deviation for the 2-min glucose concentration was relatively large. Also, this method requires a third blood sample, which would decrease the simplicity of the test, and would be more technically difficult than measurement of the fasting and 2-h samples because of the small leeway with timing.

The preferred option is using BCS to adjust 2-h glucose based on the observed effect of BCS on 2-min glucose (method a), because it is likely to provide a reasonably accurate adjustment for 2-h glucose concentrations. Although there is some error in measuring BCS, an error of 1-2 BCS units would only affect the adjusted 2-h glucose concentration by 0.10-0.2 mmol/L. Body weight is a more quantitative measure of the size of the cat than body condition score, however, the latter provides a better estimate of the extent of obesity, and had a bigger effect on many of the measured associations. Adjustments based on body weight would require knowledge of the cat’s previous ideal weight, or subjective assessment of each obese cat’s ideal weight, and this might be less accurate than an assessment of body condition score. However, adjustment on body weight could be useful in research or clinical practice where the actual increase in body weight above normal was known, or morphometric measures were used to calculate ideal body weight\textsuperscript{39,40}. The alternative strategy proposed is to adjust the dose rate, with the aim of reducing the peak glucose concentration in obese cats to the same or similar to that in lean cats. This theoretical approach to calculation of the reduced dose rate
needs to be confirmed through an empirical study to identify the correct dose rate for different body condition scores in cats. The calculations used were theoretical and several assumptions were made, but regardless of these limitations, the spurious effect is likely small, particularly in relation to the magnitude of the precision error, and only of concern in an obese cat that is close to the cutpoint.

Our study has provided an important foundation for the development of a feline equivalent to the human GTT for identifying glucose intolerance in cats. The feline test includes glucose being administered intravenously, but would require only 2 blood samples, and would provide clinical researchers and veterinary practitioners with a practical screening tool for prediabetes in cats. We propose that a standard reference range for 2-h glucose be developed to interpret GTT results for cats of all body condition scores, and this range should be determined from cats with ideal BCS (4 and 5). Testing of overweight and obese cats would then require an adjustment to compensate for the ‘dosage’ effects of obesity, following guidelines outlined in this research.

The main limitations in this study are related to the fact that the study population was young adult cats less than 4 years of age, whereas glucose tolerance testing to identify prediabetic cats will have most use in senior cats because of the age predisposition to diabetes in this species. It is recommended that a longer fasting period before testing be used if cats are fed once a day with a high carbohydrate diet (50% of energy), because blood glucose concentrations may take 24 h to return to baseline in some cats. Withholding food for 14 h is sufficient for 75% of cats fed twice daily a high carbohydrate diet. Another limitation is that data was adjusted based on assumptions rather than actual measurements and the data quality could be improved by using real data in future studies such as by titrating the initial glucose concentrations during the GTT to the same levels of glycaemia in obese and lean
cats. These data was collected retrospectively and thus validation of the assumptions was not possible. However, this is a potential area for future studies.

In conclusion, dosing obese cats based on body weight spuriously affects some measures of glucose tolerance in a glucose tolerance test, and could lead to cats being incorrectly classified as having impaired glucose tolerance. For an individual cat, the dosing effect is relatively small, and only of concern if 2-h blood glucose concentration is just above the upper cutpoint of the reference range – the maximum expected increase in 2-h glucose concentration is 0.4 mmol/L for obese cats (BCS 9 on a 1-9 scale). However, it has important implications for clinical studies assessing the effect of interventions such as diet or medication on glucose tolerance, if glucose concentrations are compared between lean and obese cats. This dosing effect may be accounted for in overweight and obese cats by adjusting down either the dose of glucose administered by 0.05 g for every kg increase above ideal weight, or the measured 2-h glucose value by 0.1 mmol/L for every unit of BCS increase above 5 on a 9-point scale, before interpretation with standardised reference limits.
### Tables

**Table 4.1** Regression coefficients, 95% confidence intervals and \( P \)-values for the associations between body weight and body condition score and peak, 2-min, and 2-h blood glucose concentrations, and half-life (\( T \frac{1}{2} \)) following glucose bolus of 0.5 g/kg body weight for 16 healthy cats assessed before and after a 9 to 12 mo ad libitum feeding period.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Peak blood glucose</th>
<th>2-min blood glucose</th>
<th>( T \frac{1}{2} )</th>
<th>2-h blood glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>For every extra kg body weight, the estimated increase was 1.50 mmol/L (95% CI 0.40 to 2.61; ( P = 0.008 )).</td>
<td>For every extra kg body weight, the estimated increase was 1.48 mmol/L (95% CI 0.27 to 2.69; ( P = 0.016 )).</td>
<td>For every extra kg of body weight, ( T \frac{1}{2} ) increases by 5.5 mins (95% CI 0.4 to 10.6; ( P = 0.034 )).</td>
<td>For every extra kg body weight, the estimated increase was 0.33 mmol/L (95% CI -0.01 to 0.67; ( P = 0.057 )).</td>
</tr>
<tr>
<td>Body condition score (BCS)</td>
<td>For every extra unit of BCS, the estimated increase was 1.06 mmol/L (95% CI 0.18 to 1.94; ( P = 0.018 )).</td>
<td>For every extra unit of BCS the estimated increase was 1.14 mmol/L (95% CI 0.19 to 2.09; ( P = 0.019 )).</td>
<td>For every extra unit of BCS, ( T \frac{1}{2} ) increases by 5.6 mins (95% CI 2.5 to 8.8; ( P &lt; 0.001 )).</td>
<td>For every extra unit of BCS, the estimated increase was 0.32 mmol/L (95% CI 0.11 to 0.53; ( P = 0.003 )).</td>
</tr>
</tbody>
</table>
**Table 4.2:** Body condition scores (BCS), fasting blood glucose concentrations, T $\frac{1}{2}$ values, and measured and adjusted 2-h blood glucose concentrations after a 9 to 12 mo *ad libitum* feeding period in 16 cats. Cats with suspected glucose intolerance based on T $\frac{1}{2}$ values are bolded.

<table>
<thead>
<tr>
<th>Cat</th>
<th>BCS</th>
<th>Fasting blood glucose concentration mmol/L</th>
<th>T $\frac{1}{2}$ after weight gain (upper limit of normal reported as 74[15] and 95[7] min)</th>
<th>Measured 2-h blood glucose mmol/L</th>
<th>Adjusted 2-h blood glucose mmol/L using method a*</th>
<th>Adjusted 2-h blood glucose mmol/L using method b*</th>
<th>Adjusted 2-h blood glucose mmol/L using method c*</th>
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<td>105.67</td>
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<td>2.2</td>
<td>2.2</td>
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</tr>
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</table>

a*(Cat’s actual BCS – 5) x 0.10 mmol/L.

b* (Cat’s actual weight – Cat’s ideal weight) x 0.13 mmol/L

c*(Cat’s actual 2-min glucose concentration – 17.8) x 0.09 mmol/L
**Figures**

**Figure 4.1:** Causal diagram showing interrelationships between obesity and 2-min and 2-h blood glucose concentrations in cats undergoing a glucose tolerance test.

- **Obesity**
  - Direct pathological effect of obesity on 2-h glucose concentration via reduced insulin sensitivity and other mechanisms.
  - Direct causal effect of 2-min glucose on 2-h glucose: higher peak (and 2-min) glucose leads to higher 2-h glucose.

- **2-min blood glucose**
  - Effect of obesity on 2-min glucose due to dosing based on body weight regardless of body condition.

- **2-h blood glucose**
  - Indirect effect of obesity on 2-h glucose concentration due to dosing based on body weight regardless of body condition score.

**Figure 4.2:** Association between T ½ for glucose disappearance following an intravenous glucose infusion (dosed at 0.5 g/kg body weight) and each of 2-min blood glucose (A) \( r = 0.17; 95\% \text{ CI} -0.35 \text{ to } 0.62; P = 0.518 \) and peak blood glucose (B) \( r = 0.18; 95\% \text{ CI} -0.34 \text{ to } 0.62; P = 0.498 \) concentrations in 16 healthy lean cats. Lines are least squares lines of best fit.

A

B
Figure 4.3: Associations between 2-min blood glucose concentration following an intravenous glucose infusion (dosed at 0.5 g/kg body weight) and each of body weight (A) (estimated increase for each extra kg body weight 0.14 mmol/L; 95% CI 0.27 to 2.69; P = 0.016) and body condition score (B) (estimated increase for each extra unit of BCS 0.14 mmol/L; 95% CI 0.19 to 2.09; P = 0.019) in 16 healthy cats assessed before (circles) and after (triangles) approximately 10 mo of ad lib feeding. Lines are least squares lines of best fit.

A                                                                 B
**Figure 4.4:** Association between 2-h blood glucose concentration following an intravenous glucose infusion (dosed at 0.5 g/kg body weight) and each of 2-min blood glucose (A) \( r = 0.65; 95\% \text{ CI } 0.23 \text{ to } 0.87; P = 0.006 \) and peak blood glucose (B) \( r = 0.61; 95\% \text{ CI } 0.17 \text{ to } 0.85; P = 0.012 \) concentrations in 16 healthy lean cats. Lines are least squares lines of best fit.
**Figure 4.5:** Association between 2-h blood glucose concentration following an intravenous glucose infusion (dosed at 0.5 g/kg body weight) and each of body weight (A) (estimated increase for each extra kg body weight 0.33 mmol/L; 95% CI -0.01 to 0.67; P = 0.057) and body condition score (B) (estimated increase for each extra unit of BCS 0.32 mmol/L; 95% CI 0.1 to 0.5; P = 0.003) in 16 healthy lean cats assessed before (circles) and after (triangles) approximately 10 mo of ad libitum feeding. Lines are least squares lines of best fit.
References


Chapter 5: Diagnosis of prediabetes in cats: glucose concentration cutpoints for impaired fasting glucose and impaired glucose tolerance

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Short title: Prediabetes in cats

Key word: diabetes; glucose tolerance test; endocrinology; hyperglycaemia

Abstract
Diabetes is typically diagnosed in cats once clinical signs are evident. Diagnostic criteria for prediabetes in cats have not been defined. The objective of the study was to establish methodology and cutpoints for fasting and 2-h blood glucose concentrations in healthy client-owned senior cats (≥ 8 yrs) using ear/ paw samples and a portable glucose meter calibrated for feline blood. Of the 78 cats, 27 were ideal (body condition score (BCS) 4 or 5 out of 9), 31 overweight (BCS 6 or 7) and 20 obese (BCS 8 or 9); 19 were Burmese and 59 non-Burmese. Following an 18 - 24 h fast and an ear/paw blood glucose measurement using a portable glucose meter, glucose (0.5 g/kg bodyweight) was administered IV and blood glucose measured at 2 min and 2 h. Cutpoints for fasting and 2-h glucose concentrations were defined as the upper limits of 95% reference intervals using cats with BCS 4 or 5. The upper cutpoint for fasting glucose was 6.5 mmol/L. Of the overweight and obese cats, one (BCS 7) was above this cutpoint indicating evidence of impaired fasting glucose. The cutpoint for 2-h glucose was 9.8 mmol/L. A total of 7 cats (4 with BCS 8 or 9 including 1 Burmese; 3 with BCS 6 or 7, non-Burmese) were above this cutpoint and thus had evidence of impaired glucose tolerance. In conclusion, the methodology and cutpoints for diagnosis of prediabetes are defined for use in healthy cats 8 yr and older with a range of body condition scores.
Introduction

In cats, 0.2% to 1% are reported to be diabetic compared to 4 to 10% of humans. Humans with blood glucose concentrations above normal but below diabetic for fasting or at 2-h in a glucose tolerance test are classed as having impaired fasting glucose or impaired glucose tolerance respectively. They are considered prediabetic and develop diabetes at a rate of 5 - 10% per yr. It is estimated that over 50% of humans in the USA with diabetes are undiagnosed, and the number with undiagnosed prediabetes is 3 to 4 times greater than with undiagnosed diabetes. There are no corresponding data for cats in the veterinary literature. As in humans, there is a genetic predisposition for feline diabetes. Burmese cats from the United Kingdom and Oceania are approximately 4 times more likely to develop diabetes than other breeds, with one in 50 affected.

Diagnostic criteria for subclinical and prediabetes in cats have not been defined, and cats are not typically diagnosed until clinical diabetes is evident. In obese cats, mild fasting or postprandial hyperglycaemia is reported to be the only early sign of diabetes, prior to onset of classical signs of diabetes such as polyuria. Reported upper limits for normal fasting blood glucose in cats vary from 6.1 mmol/L to 9 mmol/L; this variability is due at least in part to a lack of standardization of the test protocol.

Intravenous (IV) glucose tolerance tests are used to assess glucose tolerance in cats. The ‘gold standard’ test requires multiple samples and interpretation can be difficult because of the complex calculations required to generate the necessary statistics such as glucose half-life, glucose clearance time and area under the curve. Veterinarians need screening tests for impaired fasting glucose and impaired glucose tolerance that are inexpensive, non-invasive, and easy to perform and interpret in a clinical setting. A standardized IV glucose tolerance
test would need a standardised glucose dose rate, fasting period, sampling times, and an established reference range applicable to all cats, lean, overweight and obese.

Numerous portable blood glucose meters calibrated for human blood are used for glucose monitoring in cats. Although precise, they are less accurate, typically measuring 0.5 to 2.2 mmol/L lower than a serum chemistry analyses. A meter validated for feline blood, requiring a 0.3 uL blood sample is now commercially available, facilitating successful blood sampling from the ear or foot pad and more accurate measurements. A simplified protocol for IV glucose tolerance testing in cats using this glucose meter has been reported using a glucose dose of 1g/kg, but from a practitioner’s perspective, the volume to be infused can be problematic. A glucose dose of 0.5 g/kg is typically used in cats for assessing glucose tolerance whereas 1 g/kg is used for assessing maximal insulin secretory capacity.

Administering an IV glucose dose to overweight and obese cats based on bodyweight spuriously affects some measures of glucose tolerance. This is presumed to occur because blood volume does not increase linearly with the increase in body weight due to obesity. As a result, peak (2-min) glucose concentration is higher in obese cats, which subsequently increases 2-h glucose concentration when glucose is dosed on bodyweight. This can be overcome by adjusting either the glucose dose or measured 2–h blood glucose concentration based on body condition score, so that one reference interval can be used for lean, overweight and obese cats. To the authors’ knowledge, these adjustments have not been applied to cats in the age group at risk of diabetes (≥ 8 yrs).

The aims of this study were to establish methodology and cutpoints for fasting and 2–h blood glucose concentration in healthy client-owned, senior cats of varying body condition using ear/ paw samples and a portable glucose meter calibrated for feline blood, to compare these
between Burmese and non-Burmese cats, to apply adjustment equations to 2–h blood glucose concentrations in overweight and obese cats.
Materials and Method

Study overview:

The protocol for these studies and the care and handling of these animals were approved by the Animal Experimentation Ethics Committee of the University of Queensland approval number SVS/040/10/NC/ABBOTT. In 78 client-owned cats, fasting blood glucose was measured from a paw or ear sample using a portable glucose meter then an IV glucose tolerance test performed using a glucose dose of 0.5g/kg. This was repeated in 8 of these cats 23 to 57 d later to determine variability over time. An IV glucose tolerance test using the same protocol but a glucose dose rate of 1g/kg was also subsequently performed in 11 of the 78 cats.

Animals:

Clinically healthy client-owned cats ≥ 8 yrs (n = 90) were recruited though veterinary clinics, advertisements and radio interviews between May 2011 and November 2012. Cats were tested at the University of Queensland Small Animal Clinic and a private specialist cat clinic. All cats included in the study appeared clinically healthy during examination. The cats were not on any medications except routine flea and worming control. Exclusions were based on haematological and biochemical panels, body condition score (BCS) of ≤ 3 out of a 9-point scale and behaviour of the cats. Exclusions (n = 12) were for stress/aggressive behaviour (n = 3), suspected pancreatitis based on increased fPLi of > 3.5 ug/L in line with the general interpretive guidelines of our reference laboratory (n = 2), hyperthyroidism (n = 3), ongoing health issues (n = 2), pancreatic cancer (n = 1) and BCS ≤ 3 out of 9 (n = 1). Remaining cats (n = 78) were classified as non-Burmese (n = 59) or Burmese (n = 19). Body condition scores of the cats (out of 9) included in the study were all assessed by one person (MRJ), and were 4 (8 cats), 5 (19 cats), 6 (14 cats), 7 (17 cats), 8 (14 cats) and 9 (6 cats). Data was collected on
diets of the study cats and consisted of a variety of supermarket, premium and home cooked dry and tinned food.

Protocol:

Cats were admitted to the hospital the day before the glucose tolerance tests and all cats stayed overnight. On admission, a 5-mL venous blood sample was collected for a routine health screen performed by a commercial veterinary diagnostic laboratory (Idexx Laboratories, Brisbane Australia). The following morning, after food was withheld for 18 to 24 h, a jugular venous blood sample (4 mL) was collected for hormone assays and then a 22-gauge catheter (Surflo 22G x1” intravenous catheter, Terumo Europe, Belgium) was placed in the cephalic vein and flushed (2 mL 0.9% sodium chloride (Baxter)). To allow for resolution of stress hyperglycemia, fasting blood glucose was measured 3 h after catheter placement. A portable glucose meter calibrated for feline blood (AlphaTRAK) was used and the sample obtained from the paw or ear. Glucose (undiluted 50% glucose injection BP; Astra Pharmaceutical) (0.5g/kg) was then administered IV over 30 s via the catheter. A timer was started halfway through the infusion and blood samples were taken at 2 min, 2 h and then hourly until glucose returned to below our laboratory’s upper limit of normal fasting glucose concentration of 6.5 mmol/L. On completion, the catheter was removed, cats were fed and discharged.

Blood samples from syringes from 3 cats were analysed 20 times with 2 different portable glucose meters of the same brand within 1 h of collection to assess intra and inter-meter variability. The interassay CV for the glucose meter was 2 % and the intra-assay 3.3 %. To determine repeatability, fasting blood glucose assessments and glucose tolerance tests were repeated in 8 cats 23 to 57 d after their first admission (median 42 d). To compare the previously-derived adjustment equations with those derived from this population of cats, a
glucose tolerance test using the same protocol but a glucose dose rate of 1g/kg was also performed in 11 of the 78 cats (BCS 4 n = 3; 5 n = 3; 7 n = 4; 8 n = 1) 38 to 365 d later (median 60 d), depending on client availability, after their first glucose tolerance test.

Statistical analyses:

Reference intervals for fasting and 2-h glucose concentration were calculated using published method used in humans, whereby data are transformed as necessary and outliers identified and excluded from analysis\textsuperscript{26}. This methodology results on average in a 10 % narrower reference interval than if outlier detection was not used\textsuperscript{27}. Data were entered into a spreadsheet (Microsoft Excel, Reference Interval Draft Version, Copyright 2005, University of Cincinnati), transformed to approximate a normal distribution using the Box-Cox transformation, and outliers excluded from subsequent calculations. Diagnostic cutpoints were defined as the upper limits of the 95% reference intervals. Associated 90% confidence intervals (CI) for the upper limits of the reference intervals were estimated using bootstrapping with 1000 replications. Based on a priori knowledge that some overweight and obese cats have abnormal glucose tolerance\textsuperscript{15}, only lean cats (BCS of 4 or 5) were used for estimating fasting and 2–h reference intervals. Data from Burmese were pooled with non-Burmese to determine reference intervals for fasting and 2-h glucose concentrations as the median glucose concentrations and interquartile ranges were similar (median fasting Burmese and non-Burmese 4.6 and 4.7 mmol/L, respectively, and 0.7 and 1.1 mmol/L respectively; median 2-h Burmese and non-Burmese 6.2 and 5.7 mmol/L respectively, and interquartile range 2.6 and 3.1 mmol/L, respectively.
Repeatability was established using repeatability coefficients calculated using specialized software (the Pairs etc module (version 3.57) of the WinPepi software (version 11.62; www.brixtonhealth.com)).

Repeatability coefficients were calculated:

based on the within-cat variance. Approximate 95% CIs were obtained by substituting confidence limits for the within-cat variance, estimated by the method described by Zar\textsuperscript{28} (formula 7.16).

Associations between breed (Burmese or non-Burmese) and each of 2-min and 2-h glucose concentrations were assessed using linear regression with body condition score, age (both fitted as continuous variables) and sex (fitted as covariates). Associations between body condition score and 2-min glucose concentration, 2-min and 2-h glucose concentration and fasting and 2-h glucose concentrations were each assessed using univariable linear regression. Homoscedasticity of residuals were assessed using plots of residual versus fitted values. The effects of glucose dose on 2-h glucose concentration were also assessed using linear regression, with cat-time as the unit of analysis, with cat fitted as a random effect; maximum likelihood estimation was used. Interactions between dose and each of breed (Burmese or non-Burmese) and body condition score (fitted as a continuous variable) were also assessed. Regression analyses were performed using a commercial software program (Stata (version 12, StataCorp, College Station, Texas, USA)).

Adjustments of measured 2-h glucose:

We used two previously developed algorithms (Reeve-Johnson et al, unpublished data), to compensate for the spurious effect on 2-h glucose concentration that arises from dosing on a bodyweight basis (rather than using total blood volume), as previously demonstrated in obese dogs\textsuperscript{22}. Using one algorithm, observed 2-h glucose concentration was adjusted downward by
0.1 mmol/L for every unit of BCS above 5. Using the other algorithm, the difference between the observed 2-min blood glucose concentration and the mean 2-min blood glucose concentration of lean cats (17.5 mmol/L) was calculated, and multiplied by 0.09. The measured 2-h blood glucose concentrations were then adjusted downwards by subtracting the calculated product; this was done for all cats with values above the upper cutpoint.
Results

Fasting blood glucose concentrations

The upper cutpoint for fasting blood glucose concentration in cats with BCS 4 and 5 (n = 27) was 6.5 mmol/L based on the upper limit of the 95% reference interval (Table 5.1). When the statistical power was increased by including all 78 study cats (BCS varied from 4 to 9), the upper cutpoint was 6.3 mmol/L and the 90% CI 6.0 to 6.5 mmol/L. Only 1 of the 51 cats (2%) with BCS 6 to 9 was classed as having impaired fasting glucose (> 6.5 mmol/L) based on this cutpoint (BCS 7; non-Burmese), as well as one of the lean cats (BCS 5; non-Burmese). The lower limit of the 95% reference interval for cats with BCS 4 and 5 was 3.9 mmol/L (90% CI 3.6 to 4.2 mmol/L), and when all 78 cats were included, was 3.4 mmol/L (90% CI 3.2 to 3.5 mmol/L).

When 8 lean cats were retested 23 to 57 d later, the repeatability coefficient for fasting blood glucose concentration was 1.1 mmol/L (95% CI 0.7 to 2.2 mmol/L) when data from 7 of the 8 cats were used. One cat had an initial value of 4.6 mmol/L, and a value of 12.3 mmol/L after a further 43 d. At the first and second tests, fasting blood glucose concentrations for the other 7 cats ranged from 3.6 to 5.6 mmol/L and 4.1 to 5.7 mmol/L, respectively. When this cat was included in the data, the repeatability coefficient was 5.4 mmol/L (95% CI 3.7 to 10.4 mmol/L). As the 95% CI for these repeatability coefficients was wide, this estimate should be interpreted with caution. The second value for this latter cat was inconsistent with fasting concentrations in healthy cats and may have been the result of stress hyperglycaemia or laboratory error such as a bubble in the blood sample. These results indicate that when cats are tested twice 23 to 57 d apart, glucose concentrations differ within cats by up to about 1.1 mmol/L for most cats.
2-h blood glucose concentrations

The cutpoint for 2-h blood glucose concentration in an IV glucose tolerance test using 0.5 g/kg glucose estimated from cats with BCS 4 or 5 (n = 27) was 9.8 mmol/L. This was the upper limit of the 95% reference interval (90% CI 8.5 to 10.7 mmol/L) (Table 5.1). The repeatability coefficient for 2-h blood glucose concentration was 3.8 mmol/L (95% CI 2.6 to 7.2 mmol/L).

Adjustment for effect of BCS on interpretation of glucose tolerance test results

The measured 2-h blood glucose concentration for cats in the present study was adjusted in overweight and obese cats (BCS > 5) using 2 previously established algorithms (Reeve-Johnson et al, unpublished data), and the adjusted values compared to the upper cutpoints established in the present study. A total of 7 cats had 2-h glucose concentrations above the diagnostic cutpoint reported above of 9.8 mmol/L (4 obese (BCS 8 or 9), 3 overweight (BCS 6 or 7); 5 domestic, 1 Burmese and 1 British Blue). Adjusted 2-h blood glucose concentrations from both algorithms for these 7 cats were all above the upper limit of the reference range, and thus all were considered to be glucose intolerant (Appendix tables 5.3 and 5.4).

Effect of breed on fasting and 2-h blood glucose concentration

Although Burmese cats are overrepresented amongst diabetic cats, after adjusting for BCS, sex and age, Burmese cats (n = 19) did not have significantly differing fasting and 2-h glucose concentrations compared to non-Burmese (n = 59) cats. After adjusting for BCS, sex and age, the estimated difference in mean 2-h blood glucose concentrations (Burmese minus non-Burmese) was -0.6 mmol/L (95% CI of difference -1.4 to 0.2; P = 0.140). After adjusting
for BCS, sex and age, the estimated difference in mean 2-h blood glucose concentrations (Burmese minus non-Burmese) was 0.1 mmol/L (95% CI of difference -1.1 to 1.3; P = 0.856).

*Associations between body condition score and 2-min glucose concentration, and 2-min glucose and 2-h glucose concentrations*

There tended to be a positive association between 2-min glucose concentration and body condition score; for every 1 unit increase in body condition score, 2-min glucose concentration increased by 0.8 mmol/L (95% CI -0.1 to -1.7 mmol/L; P = 0.078). There was no significant association between 2-min and 2-h glucose concentrations (P = 0.396) but the point estimate was consistent with a positive relationship; for every 1 mmol/L increase in 2-min glucose concentration, 2-h glucose concentration increased by 0.04 mmol/L (95% CI -0.054 to -0.14). Although, these point estimates were not significantly associated, they were of similar magnitude to previously determined adjustments in another cohort of cats (Reeve-Johnson *et al*, unpublished data).

*Effect of glucose dose rate on 2-h blood glucose concentrations*

We evaluated the effect of glucose dose (0.5 versus 1.0 g/kg bodyweight) on 2-h blood glucose concentrations in lean, overweight and obese cats (n = 11; BCS 4 n = 3; 5 n = 3; 7 n = 4; 8 n = 1). Increasing the dose rate from 0.5 g/kg to 1 g/kg increased 2-h glucose in non-Burmese cats by an estimated 1.4 mmol/L (95% CI -0.1 to 2.8; P = 0.031). However, in Burmese, relative to 0.5 g/kg, 1 g/kg had a much larger effect; 2-h glucose was 6.4 mmol/L higher than for the lower glucose dose (95% CI 4.6 to 8.1; P < 0.001; P for interaction 0.001). Mean 2-h glucose concentration for Burmese was estimated to be 0.7 mmol/L lower than for non-Burmese (95% CI 1.2 lower to 2.6 higher; P = 0.483) at 0.5 g/kg but 5.6 mmol/L higher
(95% CI 3.7 to 7.5; $P < 0.001$) at 1 g/kg. No significant interaction was detected between dose and BCS ($P$ for interaction 0.334). Increasing the dose rate from 0.5 g/kg to 1 g/kg increased 2-h glucose by an estimated 2.2 mmol/L (95% CI -0.4 to 4.9; $P = 0.098$) where BCS was 4, and by an estimated 4.5 mmol/L (95% CI 1.4 to 7.7; $P = 0.005$) where BCS was 8.

**Associations between fasting glucose concentration and 2-h glucose concentrations**

We assessed whether there was an association between fasting glucose and glucose concentrations at 2-h in an IV glucose tolerance test, because cats with impaired fasting glucose might be expected to also have impaired glucose tolerance. For every unit increase in fasting glucose, 2-h glucose increased by 0.5 mmol/L ($P = 0.0064$; 95% CI 0.2 to 0.9). Two cats of BCS 5 and 7 had high fasting glucose concentrations (>10 mmol/L) and this positive relationship between fasting and 2-h glucose was almost entirely due to these cats.
**Discussion**

In this study of cats 8 yr or older, we established a standardized clinical protocol for diagnosing impaired fasting glucose and glucose tolerance using a portable glucose meter. The upper cutpoint for normal fasting glucose concentration was 6.5 mmol/L and for 2-h glucose concentration following a simplified IV glucose tolerance test (delivering 0.5 g/kg glucose dose) was 9.8 mmol/L. When applied to cats with a range of body condition scores, 3% were classed as having impaired fasting glucose and 9% as glucose intolerant. In contrast, 12 to 26% of human populations in USA, Europe and Australia have impaired fasting glucose and 7 to 28% are reported to be glucose intolerant. However, reported rates of overweight and obesity are typically higher in these human populations (66-75%) than are reported from feline studies (14-32 - 63%33), although the rate in cats varies with the population studied, and how body condition was measured33,34. In the absence of more accurate data on the frequency of prediabetes in the feline population 8 yrs of age or older, it is unknown if more stringent cutpoints should be applied, for example, 90% reference intervals or lower. For fasting glucose, the 90% interval would result in an upper cutpoint of 6.2 mmol/L. In humans, a link between microvascular disease such as retinopathy and glucose concentrations35 is well accepted. As this link has not been established in cats, we have chosen to use the 95% reference intervals.

Currently, there is no accepted cutpoint between impaired fasting glucose and diabetes in cats and various values have been suggested ranging from 9.536 to 16 mmol/L, with the latter approximately representing the renal threshold14. In humans, cutpoints were established in part based on the association with renal and microvascular complications6. There is an urgent need for these cutpoints to be established in cats, especially for fasting glucose, because this measurement is easily evaluated in clinical practice. The prevalence of undiagnosed diabetes
in adults in a U.S. population was 2.8%, increasing to 5.8% by the age of 60 yrs\textsuperscript{37}. It is unknown how many cats have undiagnosed diabetes. Until the cutpoint for diabetes is established, the authors suggest using 6.5 mmol/L as the upper cutpoint for impaired fasting glucose, and unstressed cats with glucose concentrations of ≥10 mmol/L that are confirmed with repeated measurements be considered diabetic\textsuperscript{38}.

Humans with impaired fasting glucose or impaired glucose tolerance are considered prediabetic\textsuperscript{6,29,30}, because they are at high risk of developing diabetes, with 5-10% of individuals progressing to diabetes per yr\textsuperscript{35}. Evidence-based cutpoints are important for diagnosing prediabetes in at risk cats, such as obese and Burmese cats. Because cats with impaired fasting glucose or glucose intolerance are at increased risk of diabetes\textsuperscript{7}, prediabetic cats need to be identified, and management regimes implemented including weight loss and dietary intervention.

\textit{Repeatability of fasting blood glucose concentrations}

Repeatability coefficients describe repeatability from a clinical perspective, ie. if the same animal is sampled on different day, how much variation is likely between two results. This incorporates both the within lab precision plus the biological variation within the same animal. Repeatability studies showed that fasting glucose concentrations differed within cats over 3-7 weeks by approximately 1.0 mmol/L for most cats. The group size, the heterogeneity and the lack of acclimatization would have contributed to the relatively large variation. Diagnosis of impaired fasting glucose or impaired glucose tolerance in humans is based on the mean of two values measured no more than 3 months apart\textsuperscript{6,30}, and a similar recommendation would be prudent for cats.
Reference values for 2-h blood glucose concentrations

Our upper cutpoint for 2-h glucose concentration of 9.8 mmol/L was similar to 9.5 mmol/L established previously by Link et al\textsuperscript{14}, but higher than 6.0 mmol/L calculated from Appleton’s raw data\textsuperscript{39} (data not shown), and likely higher than estimated from Hoenig’s\textsuperscript{15} lean cats (mean concentration estimated from graph was 5.6 mmol/L. The latter two studies used acclimatized research cats, and inserted jugular catheters under general anesthesia prior to obtaining blood samples, decreasing the probability for stress hyperglycaemia. They also used automated analysers which delayed sample analysis and might have contributed to lower glucose concentrations. Link et al\textsuperscript{14} used human portable glucose meters calibrated for whole blood which are biased to lower readings than meters calibrated for cat blood that provide plasma-equivalent measurements\textsuperscript{20}. Appleton’s cats were much younger (1-5 yrs old) and there is some evidence glucose tolerance decreases with age in cats\textsuperscript{40}.

Results from an IV glucose tolerance test is more sensitive (but slightly less specific) than fasting blood glucose for identifying people at high risk of diabetes\textsuperscript{30}. Reflecting this higher test sensitivity, impaired glucose tolerance is more prevalent than impaired fasting glucose in human populations\textsuperscript{30}. Similarly, in our study, 9\% of all cats and 20\% of obese cats had impaired glucose tolerance, whereas only 3\% of overweight cats (BCS 6–7), and no obese cats had impaired fasting glucose. We tested only cats $\geq$ 8 yr old and recruited a large proportion (65\%) that were overweight or obese, because this age group and body condition are at greatest risk of developing diabetes. Also, glucose tolerance decreases with age and increasing body condition\textsuperscript{15,41}. The prevalence of abnormal glucose homeostasis would be expected to be lower if all ages or more lean cats had been included.
**Repeatability for 2-h blood glucose concentrations**

Based on our results, there is a 95% expectation that two measurements would differ within cats by less than 3.8 mmol/L but by as much as 7.2 mmol/L. Caution is necessary when interpreting a single test result in client-owned cats because compared to acclimatized cats, non-acclimatized cats have a longer glucose half-life, attributed to stress\(^42\). Struggling 10 min prior to blood sampling is reported to increase blood glucose by as much as 10 mmol/L in cats\(^24\). We recommend retesting cats with glucose concentrations above the cutpoints, based on the variability of glucose tolerance test results in humans\(^43-45\) and cats\(^42\), although owner compliance may limit retesting for client-owned cats.

**Effect of breed on fasting and 2-h blood glucose concentrations and dose**

Neither fasting nor 2-h blood glucose concentrations were higher in Burmese compared to non-Burmese cats. Despite this, Burmese are 3 to 4 times more likely to develop diabetes than non-Burmese cats\(^46\). Because Burmese had significantly higher 2-h blood glucose concentrations at the higher dose rate, it could suggest relative intolerance to glucose at higher doses and this warrants further investigation.

**Protocol standardization**

The glucose dose rate used for a glucose tolerance test depend on the measurements of interest. In cats, 1 g/kg is more sensitive than 0.5 g/kg for determining abnormalities in insulin secretory patterns and maximum insulin secretory capacity\(^15\). However, a lower glucose dose rate (i.e. 0.5 g/kg) is used when investigating insulin action\(^14,39\). Our study used a glucose dose rate of 0.5 g/kg. The higher dose of 1 g/kg was observed to cause nausea and distress in some cats (personal observations Reeve-Johnson and Gottlieb) and the lower dose
rate (and therefore volume of injection) was considered more user-friendly for practitioners. However, at 1 g/kg, the significantly higher 2-h glucose concentrations in Burmese compared to non-Burmese cats raises the question whether a higher glucose dose can better differentiate cats with impaired glucose tolerance.

Our aim was to establish reference intervals for use in veterinary practice. Our protocol decreases technical and laboratory variability reported to affect measured blood glucose concentrations\textsuperscript{15}. The same type of portable glucose meter can be used in each veterinary practice to measure glucose immediately after blood collection, avoiding the variable time delay in measuring glucose using a variety of serum chemistry analysers in external laboratories. Postprandial glucose concentrations can be strongly influenced by diet\textsuperscript{47} and thus blood glucose should be measured in fasted cats. This requires a 14-h fast if less than 50\% of the daily energy requirement is consumed, and a 24-h fast after 100\% of the daily energy requirement is consumed\textsuperscript{48}. In our study, cats were fasted for 18-24 h and hospitalized overnight to avoid owner non-compliance and to minimize confounding of blood glucose measurement by stress.

\textit{Associations of 2-min and 2-h glucose concentrations and adjustment for obesity}

Adjustment for the spurious effects of obesity on glucose measurements following glucose dosing based on body weight was further evaluated in this study. While the associations between 2-min and 2-h glucose concentrations were not significant in the present study compared to our previous study (Reeve-Johnson et al, unpublished data), the calculated values for adjustment were very similar to those previously reported (0.05 versus 0.09 mmol/L per unit of body condition above 5; $P = 0.282$ versus $P = 0.006$ respectively). Hence, any cat with a BCS $\geq 6$ which is persistently just above the cutpoint at 2 h should have the observed glucose concentration adjusted downward by 0.1 mmol/L per unit of BCS above 5.
The 2-min blood sample following the glucose injection was difficult to obtain with accurate timing using a lancing device on the ear using one veterinarian and one handler. Adjusting on BCS is more precise (Reeve-Johnson et al, unpublished data), and it is therefore recommended.
Conclusion

We have established the methodology and cutpoints for fasting glucose and glucose tolerance in a simplified intravenous glucose tolerance test for identifying prediabetic cats in clinical practice with lean or obese body condition. We recommend 6.5 mmol/L for the cutpoint between normal and impaired fasting glucose, and 9.8 mmol/L for the 2-h glucose cutpoint between normal and impaired glucose tolerance when using a glucose dose of 0.5g/kg with blood glucose measured from ear or pad samples using a portable glucose meter calibrated for feline blood and performed after an overnight fast and hospitalization. Impaired fasting glucose and glucose intolerance should be confirmed by repeat measurements, to minimize the probability of incorrectly diagnosing a cat with stress hyperglycaemia as prediabetic.

Using the criteria established, 20 % of obese cats 8 yr of age or older are glucose intolerant. Prospective studies are required to determine the relative risk of diabetes in cats with glucose concentrations above these cutpoints. It is recommended that measured 2-h glucose concentration be adjusted downward by 0.1 mmol/L for every BCS above 5, and tests be repeated to confirm abnormal glucose tolerance.

Table 5.1: Descriptive statistics and upper limits of 95 % reference intervals (90 % confidence intervals) in mmol/L after fasting, and 2 min and 2 h after a glucose infusion of 0.5 g/kg bodyweight iv for all cats (n = 78) and various sub-groups; BCS was assessed using a 9-point scale.
<table>
<thead>
<tr>
<th>Sub-group of cats</th>
<th>Variables</th>
<th>Fasting blood glucose (mmol/L)</th>
<th>2-min blood glucose (mmol/L)</th>
<th>2-h blood glucose (mmol/L)</th>
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<tr>
<td></td>
<td>SD</td>
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<tr>
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<td>Range</td>
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<td>Upper limit 90% CI</td>
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<tr>
<td></td>
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<td>5.8</td>
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<tr>
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<td>34.4-37.9</td>
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1 Number of cats was insufficient to estimate reference interval
References


Chapter 6: Cutpoints for screening blood glucose concentrations in healthy senior cats

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Abstract

The aim was to determine the reference interval for screening blood glucose in senior cats, to apply this to a population of obese senior cats, to compare screening and fasting blood glucose, to assess whether screening blood glucose is predicted by breed, bodyweight, body condition score (BCS), behaviour score, fasting blood glucose and/or recent carbohydrate intake, and to assess its robustness to changes in methodology. 120 clinically healthy client-owned cats aged 8 years and older of varying breeds and body condition scores. Blood glucose was measured at the beginning of the consultation from an ear/paw sample using a portable glucose meter calibrated for cats, and again after physical examination from a jugular sample. Fasting blood glucose was measured after overnight hospitalization and fasting for 18-24 hours. The reference interval upper limit for screening blood glucose was 189 mg/dL (10.5 mmol/L). Mean screening blood glucose was greater than mean fasting glucose. Breed, bodyweight, BCS, behaviour score, fasting blood glucose concentration, and amount of carbohydrate consumed 2 to 24 hours before sampling collectively explained only a small proportion of the variability in screening blood glucose. Screening blood glucose measurement represents a simple test, and cats with values from 117 mg/dL to 189 mg/dL (6.5 mmol/L to 10.5 mmol/L) should be retested several hours later. Cats with initial screening blood glucose >189 mg/dL (10.5 mmol/L), or a second screening blood glucose >116 mg/dL (6.4mmol/L) several hours after the first, should have fasting glucose and glucose tolerance measured after overnight hospitalization.
Introduction

Diabetes occurs in 1 in 50 to 1 in 400 cats, depending on the population studied, and risk factors include age, male sex, obesity and breed. Burmese cats are overrepresented in Europe and Australasia \(^1\,2\) and Russian Blue, Maine Coon and Siamese in the USA\(^3\). The terms impaired glucose tolerance and impaired fasting glucose refer to an intermediate stage between normal glucose homeostasis and diabetes, now referred to as prediabetes\(^4\,5\). At least 60% of humans who develop diabetes have either impaired glucose tolerance or impaired fasting glucose identified in the previous 5 years\(^6\), and up to 33% of people with impaired glucose tolerance develop diabetes over a two year period\(^7\). If identified before progression to diabetes, these individuals usually return to reasonable glycaemic control with weight loss, dietary management, and exercise. As in humans, progression from impaired fasting glucose and impaired glucose tolerance to diabetes also occurs in cats. Of cats in diabetic remission with moderate impaired fasting glucose \((\geq 135-\leq 151 \text{ mg/dL}; \geq 7.5-\leq 8.4 \text{ mmol/L})\) or moderate glucose intolerance \((n=21), 75\% \text{ and } 38\%, \text{ respectively, developed diabetes within 9 months of testing}\(^8\). Impaired fasting glucose is rarely diagnosed in cats, and mild increases in blood glucose are mostly attributed to stress hyperglycaemia associated with the veterinary visit or with illness\(^6\,9\). As in humans, it is likely advantageous to differentiate cats with transient hyperglycaemia associated with stress or eating, from cats with persistent mild increases in glucose (prediabetic), so appropriate therapy can be implemented to prevent progression to overt diabetes. Measurement of fasting glucose concentration is the gold standard in human medicine used to diagnose impaired fasting glucose. However, fasting can be problematic in client-owned cats because the postprandial period in some cats fed twice daily can exceed 14 h, and 24 h in some cats fed once daily\(^10\). Although owners can fast cats at home, it does not resolve the effect of stress of travel to the clinic on blood glucose.
concentrations. Therefore, measurement of fasting blood glucose at home or in the clinic may not be a practical screening test for some pet cats.

Casual blood glucose, defined as blood glucose measured unrelated to time of eating or type of food, is used as a screening test to diagnose diabetes in humans\textsuperscript{11}. In humans, the lower cutpoint for diabetes for casual blood glucose is higher\textsuperscript{4,11} than for fasting glucose (>200 mg/dL; 11.1 mmol/L versus >126 mg/dL; 7.0 mmol/L), and requires the presence of classical signs for definitive diagnosis. It is recommended that casual blood glucose concentrations above fasting values, but unaccompanied by diabetic signs, are confirmed by measurement of fasting blood glucose and glucose tolerance testing because of the potential for confounding factors to increase blood glucose\textsuperscript{4}, such as “white coat” hyperglycaemia associated with stress\textsuperscript{12,13}.

In cats, stress hyperglycaemia is a major factor confusing diagnosis of diabetes, and struggling can increase blood glucose by as much as 180 mg/dL (10 mmol/L)\textsuperscript{6}. Cats without clinical signs of diabetes but blood glucose concentrations above the upper cutpoint for normal could have prediabetes or subclinical diabetes, or a response to stress or eating. Where other risk factors are present, such as senior age and obesity, it would be prudent to determine if the hyperglycaemia is persistent and consistent with abnormal glucose homeostasis, or transient and consistent with stress-induced hyperglycaemia or eating.

Measurement of blood glucose in capillary blood from the ear or pad using a portable glucose meter requires less restraint than for venipuncture, which may help reduce the effect of stress on measurement of blood glucose\textsuperscript{14-16}. Numerous portable blood glucose meters calibrated for human blood have been used in cats\textsuperscript{17-19} and although precise, have lower accuracy than meters calibrated for feline blood, and typically under-report blood glucose by 8-18 mg/dL (0.4-1.0 mmol/L)\textsuperscript{20}. The availability of a portable glucose meter calibrated for feline blood
(AlphaTRAK Glucose Monitoring System, Zoetis) and requiring only a small sample volume (0.3 uL)\textsuperscript{21}, facilitates more accurate measurement of blood glucose in cats.

The aims of this study were i. to establish the reference interval for a screening blood glucose test in client-owned cats aged 8 years and older, measured on entry to the clinic from a pad or paw sample using a glucose meter calibrated for cat blood (screening blood glucose), ii to apply this in a population of obese cats, iii to determine if selected variables account for variability in screening blood glucose (breed, bodyweight, body condition score, behaviour score, fasting blood glucose, recent carbohydrate intake) and iv to determine if blood glucose is affected by changes in screening blood glucose methodology that could be expected in veterinary practice, such as using a jugular sample or external laboratory.
Materials and Methods

The protocol for these studies and the care and handling of these animals were approved by the Animal Experimentation Ethics Committee of the University of Queensland approval number SVS/040/10/NC/ABBOTT.

Animals

Client-owned, neutered cats 8 years and older (mean 10.9 years, range 8-18 years; n=134, Table 6.1) presenting for a routine health check at local veterinary clinics in Brisbane were assessed. Other than for excess body condition in some cats, cats enrolled in the study were assessed as healthy based on results of a clinical examination, patient history, haematological and biochemical assays, total thyroxine, and fPLi. Body condition score (BCS) was recorded on scale of 1 to 9, and cats between 4 and 9 were included\(^\text{22}\). Exclusions (n=14) were due to chronic renal failure (n=2), hyperthyroidism (n=6), suspected pancreatitis based on increased fPLI (> 3.5 ug/L) (n=1), gastrointestinal neoplasia detected at initial examination (n=1), inability to sample from an ear or paw pad (n=1), and BCS was ≤3/9 (n=2) or not recorded (n=1). Reference intervals were established using cats with ideal body condition scores (4-5/9, n=49), and then applied in a population of otherwise healthy overweight (6-7/9, n=45), and obese (8-9/9, n=26) cats. The mean bodyweight of the 120 cats was 5.5 kg (range 2.9-10.0 kg, Table 6.1).

The following definitions for blood glucose measurement methods were used: ‘screening’ (measurement any time after eating and on entry to the consultation room prior to history taking and physical examination, from an ear or paw pad blood sample, using a portable glucose meter calibrated for cat blood), and ‘fasting’ (after overnight hospitalisation and withholding food for 18-24hr, from ear or paw pad blood sample, and measurement by glucose meter). To determine the robustness of screening blood glucose to some variations in
sampling and measurement that could be expected in a busy veterinary practice, blood glucose was also measured using a glucose meter from a venepuncture sample obtained any time after eating and after history taking and physical exam, with blood dropped directly from the syringe after removal of the needle (traditional-meter). It was then placed in a tube containing the anticoagulant sodium fluoride/potassium oxalate (FO) and measured by meter (traditional-meter-FO) and by an external laboratory in plasma (traditional-laboratory-FO).

Experimental protocol

After entry of the client and their cat into the consulting room for screening blood glucose measurement, a drop of blood was obtained from the pinna margin (n=106) or edge of the pisiform pad (n=11) using a lancet (AlphaTRAK lancing device, Zoetis). The method of sampling (pinna or pad) was not recorded for 3 cats. Whenever possible, the cat was positioned with minimal restraint with its fore-paws on the shoulder of the client. To increase perfusion, the ear/paw was warmed by holding a cotton wool ball soaked in warm water and squeezed dry to the area for approximately 10 seconds. The drop of blood was analysed immediately using the portable glucose meter using glucose oxidase methodology (AlphaTRAK, Zoetis) (screening blood glucose). Behaviour in cats were assessed by the clinician at the time of the screening blood glucose measurement and an integer score from 0 to 4 allocated based on previously determined criteria6, where 0 indicates no behaviours suggesting stress and 4 indicates behaviours suggesting extreme stress. Behaviours assessed to determine each cat’s score were escape attempts including struggling, vocalization, hypersalivation, mouth breathing, aggression and immobility.

The medical history, indoor or outdoor access, body weight (in kg), body condition score, and feeding (timing, and type and amount of food fed) were recorded, and a physical examination was performed. For 105 of the 120 study cats, a venous blood sample was taken at the end of
the consultation and measured with the meter (traditional-meter). For 48 of these, the sample was then placed into a tube containing the anticoagulant sodium fluoride/potassium oxalate and blood glucose concentration measured with the meter after using a syringe to aspirate a droplet (traditional-meter-FO), and the same tube was then sent to a commercial veterinary laboratory (Idexx Laboratories, Brisbane Australia) for blood glucose analysis with an automated serum chemistry analyser (Beckman Coulter AU 600) which used the hexokinase reaction (traditional-laboratory FO). For the portable glucose meter, the previously established inter-assay precision was 2% (coefficient of variation) and intra-assay 3.3%23. For the laboratory method, within-run precision was <3% and total precision <3%.

Study cats that were compliant and whose owner gave consent (n=74) were subsequently hospitalised overnight, and samples collected for fasting blood glucose and a simplified glucose tolerance test conducted. This was done on the day after the cat's first study visit for 51 cats and fasting values from only these cats were used in statistical analyses. Cats were fasted and glucose was measured at 0 h (fasting blood glucose) and again 2 h after receiving 0.5g/kg glucose iv, using the same pinna/ pad technique and portable glucose meter. Results from these samples are reported in a separate publication23 but comparisons between screening blood glucose and fasting blood glucose, and some individual glucose tolerance test results are reported here.

*Dietary carbohydrate calculations*

Carbohydrate consumption 2-24 h and 2-12 h prior to the screening blood glucose measurement was estimated. Carbohydrate consumed in the last two hours before sampling was not included in the calculations as blood glucose concentration would have been minimally affected by this24. Owners were informed that dietary data would be collected from them and when possible, they were emailed the data capture form prior to the consultation. Owners recorded food, amount fed and timing of feedings. Dietary composition data supplied
by the various food manufacturers were used to calculate the amount of carbohydrate (nitrogen-free extract) in grams consumed. Estimates were not generated for those cats whose owners provided incomplete or unreliable data on the food, amounts and timing of foods given/eaten by the cat (n=55 of the 120 study cats).

The nitrogen-free extract consumption of cats fed *ad libitum* (defined as food always or nearly always present, and in excess of what could be consumed) (n=39 cats) was calculated using estimated daily metabolizable energy requirements. Two methods were used:

a. Daily consumption was estimated at 60 kcal/kg/24 hours or 30 kcal/kg/12 hr.

b. Using the following formula\textsuperscript{24,25}:

\[
\text{Daily energy requirement} = 151.8 \times (\text{bodyweight (kg)})^{0.4} - 87.5.
\]

The exponent of 0.4 and the constants 151.8 and 87.5 are reported to provide an accurate estimate of daily energy requirements/kg bodyweight in cats of various body conditions from lean to obese\textsuperscript{25,26}.

Carbohydrate intake for the period (i.e. for 12 or 24 h) was then calculated for each cat as estimated daily energy intake for the period multiplied by the percentage of the dietary energy that was supplied by carbohydrate. For cats meal-fed once daily (n=26 cats), time when food was replenished was used to calculate the cats’ estimated consumption, assuming that 50% of daily energy provided was eaten in the 12 h following feeding, and 100% of daily energy provided was eaten in 24 hours, if bowls were replenished once daily. For cats fed twice or more times daily, amount fed (data provided by the owner) was used for calculation of carbohydrate intake in 12 and 24 h. These owners accurately kept a record of how much the cats were fed and at what time.

*Statistical analyses*
Reference intervals were determined after Box-Cox transformation and exclusion of outliers (Reference Interval Draft Version, Copyright 2005, University of Cincinnati). Confidence intervals for each reference interval limit were calculated using 1000 bootstrap resamplings. The upper cutpoints were defined as the upper limits of the 95% reference intervals. Medians and interquartile ranges were compared between Burmese and non-Burmese cats, and where absolute differences between the medians and interquartile ranges were <50% and <100% of the lowest median and interquartile range, respectively, the sub-groups of cats (ie. Burmese and non-Burmese) were pooled, an approach that has been used previously.\textsuperscript{27}

All other analyses were performed using a statistical software program (Stata (version 14, StataCorp, College Station, Texas, USA). Means were compared between screening and fasting samples using cats with BCS 4 or 5 whose fasting blood glucose was assessed on the day after their first study visit, using the paired t-test, calculated using Stata's -ttest- command.

The amount of variability in screening blood glucose that was accounted for by potential determinants of screening blood glucose concentration was assessed using multivariable linear regression models, simultaneously fitting breed (Burmese/Burmese cross or non-Burmese), bodyweight, BCS, behaviour score during screening blood sampling, fasting blood glucose concentration (as assessed at the cat's subsequent hospitalisation, using only those cats whose fasting blood glucose was assessed on the day after their first study visit), and amount of carbohydrate consumed in either the 2 to 24 h or 2 to 12 h before screening sampling. Behaviour score was fitted as a categorical variable with four categories (0, 1, 2, 3 and 4 combined). All six variables were recorded for only 29 of the 120 study cats but breed, BCS, behaviour score and fasting blood glucose concentration were all recorded for 49 cats. Models were fitted both including and not including bodyweight and amount of carbohydrate.
consumed. The overall P-values for behaviour score were assessed using the multiple Wald test. One cat had elevated screening and fasting blood glucose concentrations (238 and 221 mg/dL; 13.2 and 12.3 mmol/L, respectively). A further cat had lower screening but elevated fasting blood glucose concentrations (155 and 223 mg/dL; 8.6 and 12.4 mmol/L, respectively). Regression analyses were performed with and without these cats. As results were similar, only those with these cats included are reported. With preliminary univariable linear regression analyses, histograms of residuals were used to identify whether the residuals had a normal distribution. Residual-versus-predictor plots were used to assess homoscedasticity of residuals. Residuals were not normally distributed and/or were heteroscedastic in models that included the cat with elevated screening and fasting blood glucose concentrations, so the bootstrap method with 1000 replications was used for multivariable regression analyses. Normal-based confidence intervals were used.

To determine the robustness of the screening blood glucose to some changes in methodology that could be expected in veterinary practice, we compared screening blood glucose concentrations with those obtained following some changes in sampling and measurement. Agreement was assessed using Lin's concordance correlation coefficient with 95% confidence intervals based on the z-transformation, Pearson's correlation coefficient with 95% confidence intervals based on Fisher's transformation, comparison between the reduced major axis line and the line of perfect concordance, and 95% limits of agreement, using Stata's -concord- and -ci2- commands. Mean glucose concentrations were also compared, using paired t-tests, calculated with Stata's -ttest- command.

Means and standard deviations are reported, unless otherwise indicated, and P <0.05 was considered significant.
Results

Reference intervals for screening and fasting blood glucose

Distributions of screening, traditional and fasting blood glucose concentrations for the 71 cats with all three measures are shown in Figure 6.1. In this study of senior cats, using those with ideal body condition score (4 to 5/9; n=49), the reference interval for screening blood glucose was 67 to 189 mg/dL (3.7 to 10.5 mmol/L; 90% confidence interval for upper limit 164 to 212 mg/dL; 9.1 to 11.8 mmol/L). This upper limit for screening blood glucose was 73 mg/dL (4.1 mmol/L) higher than for fasting blood glucose (116 mg/dL; 6.4 mmol/L; 90% confidence interval for upper limit 107 to 120 mg/dL; 5.9 to 6.7 mmol/L) calculated using the 28 of these cats that had fasting values. For 35% (17/49) of the study cats with ideal body condition score, screening blood glucose concentration was above that upper limit for fasting blood glucose. Using only the 20 cats with ideal body condition score and whose fasting blood glucose was assessed on the day after their first study visit, mean blood glucose concentrations were significantly higher (P=0.001) for screening samples (121 ± 39 mg/dL (6.7 ± 2.1 mmol/L)) than fasting samples (96 ± 32 mg/dL (5.3 ± 1.8 mmol/L)), with a difference of 25 mg/dL (95% CI 12 to 39 mg/dL) (1.4 mmol/L; 95% CI 0.7 to 2.2 mmol/L). Behaviour scores were low (63% of cats scored 0 or 1 and a further 25% scored 2), and no cats were observed to struggle (Table 6.1).

Determinants of screening blood glucose concentration

We investigated the extent to which screening blood glucose concentration was accounted for by breed, bodyweight, BCS, behaviour score during screening blood sampling, fasting blood glucose concentration and carbohydrate intake before sampling. These six variables collectively explained only a small proportion of the variability in the screening blood glucose ($R^2$ 36%, adjusted $R^2$ 10%). This model was not significantly better than the null
model (P=0.893), and none of these variables was significantly associated with screening blood glucose after adjustment for the other five variables (Table 6.2). Results were similar when amounts of carbohydrate consumed from 2 to 12 h were used instead of 2 to 24 h before sampling. From the four-variable model with breed, BCS, behaviour score and fasting blood glucose concentration fitted, screening blood glucose concentration was also not significantly associated with fasting blood glucose concentration (Table 6.2).

The reference interval upper limit for screening blood glucose obtained from cats with ideal body condition scores (189 mg/dL or 10.5 mmol/L) was applied to the population of obese cats (range 8-9/9; n=26). No obese cats were above the upper limit. One cat (with a BCS of 5/9) had both a screening blood glucose and fasting blood glucose above the screening blood glucose cutpoint (screening blood glucose was 238 mg/dL; 13.2 mmol/L and fasting blood glucose was 221 mg/dL; 12.3 mmol/L, respectively). However, the traditional blood glucose measured 20 minutes after screening was normal (117 mg/dL; 6.5 mmol/L) and glucose tolerance the following day was normal with a blood glucose value at 2 h of 140 mg/dL; 7.8 mmol/L, which was below the upper cutpoint for our laboratory (178mg/dL, 9.9 mmol/l)\textsuperscript{23}.

Robustness of screening blood glucose to variations in methodology

Because veterinarians in practice usually take a jugular blood sample after history taking and physical examination ('traditional sampling') rather than using a screening sample, and measure blood glucose either directly from the syringe by meter (traditional-meter), or place the sample in a tube with anticoagulant (in our study, sodium fluoride/potassium oxalate) and measure with meter (traditional-meter-FO) prior to sending it to an external laboratory (traditional-laboratory-FO), we investigated how well screening blood glucose concentrations agreed with measurements of blood glucose after some changes in methods of sampling and measurement that could be expected in a busy veterinary practice (Table 6.3). Agreement
between results from screening and the three other methods was low as indicated by low Lin's concordance correlation coefficients (0.51 to 0.55) because they were not closely correlated (Pearson's correlation coefficients 0.56 to 0.58), but accuracy was high; that is, on average, results from each method from the same cat were close to each other. Differences in concentrations between screening and the three other methods were widely spread. For example, for screening versus traditional-meter, the 95% limits of agreement were -60 to +44 mg/dl (-3.4 to +2.4 mmol/L); these results indicate that for 95% of cats, the difference would fall between these values. Agreement was only a little better for each of traditional-meter and traditional-meter-FO versus traditional-lab-FO (Lin's concordance correlation coefficients 0.73 and 0.77). Concentrations were closely correlated (Pearson's correlation coefficients 0.95 and 0.96) but accuracy was poor because laboratory results were generally higher than glucose meter results. Mean differences were 17 and 14 mg/dl (0.9 and 0.8 mmol/L) lower for laboratory results compared to traditional-meter and traditional-meter-FO, respectively. Of note, when blood was dropped from the syringe with the needle attached, inconsistent and spurious results for blood glucose measurement were obtained, resulting from inconsistent mixing of red blood cells in plasma in the needle (per comm L Cozzi, Abbott Animal Health).
Discussion

In this study of healthy, client-owned, senior cats, we standardized measurement of screening blood glucose and established the upper cutpoint of 189 mg/dL (10.5 mmol/L) for ideal body condition cats using a portable glucose meter validated for feline blood (AlphaTRAK, Zoetis). Screening blood glucose was defined in our study as measurement any time after eating and on entry to the consulting room, with blood sampling from an ear or paw pad, and measurement with a portable glucose meter calibrated for cats.

The upper cutpoint for screening blood glucose was higher (73 mg/dL or 4.1 mmol/L higher) than that for fasting glucose concentration. In humans, inadequate fasting, for example less than 10 hours, can increase glucose concentrations above fasting values\textsuperscript{28}. In cats consuming a high carbohydrate diet (50% of metabolizable energy, 12.8 g per 100 kcal), blood glucose increased on average by 61 mg/dL (3.4 mmol/L) after a meal of 100% of daily energy requirements\textsuperscript{29}. Measurement of fasting blood glucose requires food to be withheld at least for 14 hours after a meal of 50% of daily energy requirements, and for 24 hours after a meal of 100% daily energy requirements\textsuperscript{10}, which may be not practical in client-owned cats. In our study, carbohydrate intake accounted for little of the variation in screening glucose, possibly because cats in our study consumed diets with moderate carbohydrate content (mean ± SD; 7.5 ± 2.4g per 100kcal) and most were fed \textit{ad libitum} or twice daily. However, there were many limitations in this calculation of carbohydrate consumed. More detailed understanding of the timing of carbohydrate consumption, along with a more detailed knowledge of the amount and nature of the carbohydrate would be required to fully evaluate effects of carbohydrate consumption. These constraints limit confidence in the findings relating to the effect of carbohydrate intake on blood glucose, and this dietary effect should be further investigated in a future study.
At the time of blood sampling, overt behaviours that may be associated with stress also did not account for the much of the variation in screening blood glucose concentrations. In a study of stress hyperglycaemia associated with a 5-min spray bath, struggling was the only behaviour associated with increased blood glucose concentration. In that study, glucose concentration increased on average by 74 mg/dL (4.1 mmol/L) within 10 minutes, and as much as 194 mg/dL (10.8 mmol/L) in some cats, and the mean change was the same magnitude to the difference between fasting and screening blood glucose in our study. The increase was associated with increased plasma lactate and norepinephrine concentrations, and resolved within 90 minutes in most cats\(^6\). However, there were limitations in our study when assessing the effect on blood glucose of behaviours previously shown to be associated with stress hyperglycaemia. Behaviour scores were low compared to cats being spray bathed, no cats were observed to struggle, our stressor was shorter and milder than in the five-minute spray bath, and in our study, non-compliant cats were excluded to facilitate successful blood collection. Because neither carbohydrate consumed nor the behavioural scores in the consulting room explained much of the variation in screening blood glucose, it is likely that this variation was, in part, the result of stress prior to entry into the consulting room, for example, stress associated with travel to the clinic. Based on continuing research in our laboratory using a continuous glucose monitor to identify when stress hyperglycaemia occurs, it is clear that travel to the clinic in some cats’ results in marked hyperglycaemia. However, not all cats in the current study had higher values for screening compared to fasting blood glucose, suggesting not all cats develop stress hyperglycaemia associated with a veterinary clinic visit, and further research is required to understand the triggers for stress hyperglycaemia.
There was no significant association between either body weight or BCS and screening blood glucose concentration, consistent with previous findings that although obesity is associated with impaired glucose tolerance, fasting blood glucose concentrations are not typically increased\textsuperscript{30,31}. This suggests that simply measuring fasting or screening blood glucose may not be sensitive in identifying cats with milder disturbances of glucose metabolism, and a glucose tolerance test should be considered if multiple risk factors for diabetes are present.

Importantly, the cutpoints were not robust to changes in methodology, and if the cutpoints determined in our study are to be used, the methodology for screening blood glucose needs to be adhered to. Compared to screening blood glucose, agreement was poor when blood glucose was measured after physical examination and jugular blood sampling using the meter with blood either dropped from the syringe or preserved with sodium fluoride/potassium oxalate then tested. There was also poor agreement with screening when glucose was measured in blood from the jugular sample several hours later by an external laboratory. There were a number of differences in methodology between screening blood glucose and these traditional sampling methods, and the relative contribution of these differences is unknown. However, they highlight the importance of adhering to the methodology described. The glycolysis inhibitor, sodium fluoride/potassium oxalate, does not completely inhibit glycolysis, and it artifactually decreases plasma glucose concentration because of movement of water from erythrocytes, resulting in plasma dilution and erythrocyte shrinkage\textsuperscript{32,33}. Therefore, if the cutpoints identified in our study are being used, the methodology needs to be the same, including collection of the sample from the ear or foot pad on entry to the clinic and before physical examination, and blood glucose measured using a portable glucose meter calibrated for feline blood immediately after collection.
A range of blood glucose cutpoints are used for diagnosing diabetes in cats, varying from 171 to 290 mg/dL (9.5 to 16 mmol/L), the latter being the renal threshold. In humans, the cutpoint for diabetes (126 mg/dL, 7 mmol/L) was established, in part, based on the associations with renal and microvascular complications. Individuals below this cutpoint but above fasting (100 mg/dL, 5.6 mmol/L) are considered prediabetic and at high risk of developing clinical diabetes. Approximately 30% of adult humans have undiagnosed diabetes, increasing to 62% by the age of 65 years. It is likely that many cats have undiagnosed subclinical diabetes, and most cats with prediabetes are undiagnosed. The blood glucose concentration cutpoint for diabetes in cats urgently needs to be established.

Based on the increased incidence of diabetes in cats 8 years of age or older, we recommend all senior cats have a screening blood glucose measured at each health check. This is especially important in cats with one or more additional risk factors for diabetes such as obesity, Burmese breed, male sex or glucocorticoid therapy. Measuring blood glucose in capillary blood from the ear or paw is easy and rapid to perform, and is less labour intensive and better tolerated than traditional methods of venous sampling (personal observation of author, Reeve-Johnson). In our study of 134 cats, a screening blood sample was easily obtained in all but 1 cat and, for that cat, sampling from an ear or paw pad was successfully performed at a later date.

If blood glucose during the screening test is higher than the upper cutpoint for fasting (116 mg/dL; 6.5 mmol/L) but lower than the upper cutpoint for screening (189 mg/dL; 10.5 mmol/L), we recommend the cat is retested 3-4 hours later to determine if the hyperglycaemia has normalized and was likely associated with stress or eating. If hyperglycaemia is persistent, the cat should be retested at home or after overnight fasting and hospitalisation, ideally with a glucose tolerance test. In one study, stress hyperglycaemia resolved within 90 minutes after the end of the stressor in the majority of cats, although in
8/20 cats, glucose concentrations were above baseline (range > 83 mg/dL; 4.6 mmol/L - 258 mg/dL (14.3 mmol/L), and in general, these cats had higher peak glucose concentrations during the spay bath⁹. In the author’s (JR) experience, 3-4 hours is sufficient for stress hyperglycaemia to resolve in the majority of cats if they are quietly housed, and the second sample from the ear or pad is collected with the cat in the cage or carry basket. There is lack of definitive research in this area, but stress hyperglycaemia is likely affected by the nature and duration of the stressor(s) and the individual cat.

Senior cats with an initial screening blood glucose > 189 mg/dL (10.5 mmol/L) should have fasting blood glucose and glucose tolerance measured after an 18-24 hr fast and overnight hospitalisation. Based on data in humans¹¹, senior cats with persistent mild hyperglycaemia or glucose intolerance could be considered prediabetic and at risk of developing diabetes, especially if other risk factors are present. Cats in diabetic remission with persistent fasting blood glucose between 135 mg/dL (7.5 mmol/L) and 151 mg/dL (8.4 mmol/L) have an estimated 14 times higher odds of development of diabetes compared to those whose glucose is less than 135 mg/dL (7.5 mmol/L). Mild persistently increased fasting blood glucose is therefore likely to be an indicator or risk factor for diabetes in other groups of predisposed cats, such as senior obese or Burmese cats⁸. A longitudinal study would be required to identify the percentage of cats with impaired fasting glucose or glucose intolerance which develop clinical diabetes within 1 to 3 years.

One study cat had conflicting blood glucose data with screening 238 mg/dL (13.2 mmol/L) and a behaviour score of 2; traditional-meter 117 mg/dL (6.5 mmol/L); fasting 221 mg/dL (12.3 mmol/L) and a normal glucose tolerance test. In humans and cats, glucose tolerance testing is more sensitive at detecting abnormalities of glucose metabolism than measurement of fasting glucose⁸. While occasionally some humans have mild impaired fasting glucose and normal glucose tolerance, this is unusual³⁹. In this cat, screening and fasting blood glucose
were in the diabetic range\textsuperscript{40} and but traditional blood glucose measured approximately 20 minutes after screening glucose was normal, and glucose tolerance was normal measured the next day, suggesting both screening and fasting samples were stress affected. No clinical signs of diabetes developed in the ensuing 4 years after testing, supporting this interpretation of results. When fasting glucose is increased, but glucose tolerance is normal, stress hyperglycaemia should be suspected and measurement of blood glucose at home be considered.

In prediabetic humans, changes in lifestyle which increase physical activity and decrease bodyweight can mitigate the risk of developing diabetes\textsuperscript{41}. In senior cats with impaired fasting glucose or impaired glucose tolerance, especially if other risk factors are present, it would also be prudent to implement strategies to reduce risk. For example, implementing a weight loss protocol and increasing physical activity in obese cats, changing from a high carbohydrate to a low carbohydrate diet, and reducing or eliminating steroid administration\textsuperscript{42}.
**Conclusions**

We established cutpoints for screening blood glucose concentrations in a population of healthy cats aged ≥ 8 years and demonstrated that cutpoints are not robust to changes in methodology. We recommend that screening blood glucose, as described in our study, be included in the health examination for all senior cats. It is simple to perform, is well tolerated, and has the benefit of only needing 0.3 uL of blood. Cats with risk factors for diabetes and abnormal results should be retested to confirm they are persistent. For cats with persistently increased fasting glucose (impaired fasting glucose) or impaired glucose tolerance, it would be prudent to implement management strategies to reduce risk of future diabetes, especially in cats with reversible risk factors such as obesity and corticosteroid administration. In cats where stress hyperglycaemia is suspected, it may be advisable to measure blood glucose at home. Future studies are needed to determine the relative risk for diabetes of blood glucose concentrations persistently above the cutpoints identified in this study, and to better understand transient hyperglycaemia in cats associated with stress and eating.
<table>
<thead>
<tr>
<th></th>
<th>BCS/9 (mean±SD) (range)</th>
<th>Bodyweight (kg) mean±SD (range)</th>
<th>Age (years) mean±SD (range)</th>
<th>Sex (F/M)</th>
<th>Burmese and Burmese cross/non-Burmese (B/NB)</th>
<th>Behaviour score % ≥2 (range)$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>All cats n=120$^2$</td>
<td>6.2±1.5 (4-9)</td>
<td>5.5±1.6 (2.9-10.0)</td>
<td>10.9±2.4 (8-18)</td>
<td>62F 58M</td>
<td>29B 91 NB</td>
<td>34% (0-4)</td>
</tr>
<tr>
<td>Ideal body condition cats (body condition scores 4 or 5) n=49$^2$</td>
<td>4.7±0.5 (4-5)</td>
<td>4.3±0.7 (2.9-5.5)</td>
<td>11.4±2.6 (8-18)</td>
<td>22F 27M</td>
<td>12B 37NB</td>
<td>35% (0-4)</td>
</tr>
<tr>
<td>Overweight cats (body condition scores 6 or 7) n=45$^2$</td>
<td>6.6±0.5 (6-7)</td>
<td>5.5±1.0 (4.1-7.3)</td>
<td>10.8±2.3 (8-17)</td>
<td>24F 21M</td>
<td>15B 30NB</td>
<td>26% (0-3)</td>
</tr>
<tr>
<td>Obese cats (body condition scores 8 or 9) n=26$^2$</td>
<td>8.3±0.5 (8-9)</td>
<td>7.4±1.2 (5.6-10.0)</td>
<td>10.2±2.2 (8-16)</td>
<td>16F 10M</td>
<td>2B 24NB</td>
<td>46% (0-3)</td>
</tr>
</tbody>
</table>

$^1$For all groups, the median score was 1
$^2$For bodyweights, ages, and behaviour scores, numbers of cats are less than shown for some groups as these data were not recorded for all cats
Table 6.2 Regression coefficients (estimated changes in screening blood glucose in mg/dL (mmol/L)), 95% confidence intervals for estimates and p-values for the associations between screening blood glucose and each of breed, bodyweight, body condition score, behaviour score during screening blood sampling, fasting blood glucose concentration and carbohydrate intake from 2 to 24 hr before sampling for healthy cats ≥8 years of age

<table>
<thead>
<tr>
<th>Variable and category</th>
<th>Adjusted regression coefficient</th>
<th>95% confidence interval</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breed (Burmese/Burmese cross relative to non-Burmese)</td>
<td>-8.0² (−0.4)</td>
<td>−58.0 to 42.0</td>
<td>0.754</td>
</tr>
<tr>
<td>Bodyweight (kg)</td>
<td>−7.1³ (−0.4)</td>
<td>−31.4 to 17.3</td>
<td>0.570</td>
</tr>
<tr>
<td>Body condition score</td>
<td>9.9 (0.6)</td>
<td>−15.8 to 35.7</td>
<td>0.450</td>
</tr>
<tr>
<td>Behaviour score:</td>
<td></td>
<td></td>
<td>0.260³</td>
</tr>
<tr>
<td>1 relative to 0</td>
<td>31.1 (1.7)</td>
<td>−7.7 to 69.8</td>
<td>0.116</td>
</tr>
<tr>
<td>2 relative to 0</td>
<td>15.1 (0.8)</td>
<td>−25.2 to 55.5</td>
<td>0.463</td>
</tr>
<tr>
<td>3 or 4 relative to 0</td>
<td>50.6 (2.8)</td>
<td>−8.9 to 110.1</td>
<td>0.095</td>
</tr>
<tr>
<td>Fasting blood glucose concentration (mmol/L)</td>
<td>11.8⁵,⁶ (0.7)</td>
<td>−13.3 to 37.0</td>
<td>0.355</td>
</tr>
<tr>
<td>Carbohydrate intake from 2 to 24 hr before sampling (g)</td>
<td>−0.2⁷ (0.0)</td>
<td>−4.8 to 4.4</td>
<td>0.925</td>
</tr>
</tbody>
</table>

¹Adjusted for the other five variables in the model
²Screening blood glucose concentration was estimated to be 8 mg/dL (0.4 mmol/L) lower in Burmese/Burmese cross relative to non-Burmese (95% confidence interval 58.0 mg/dL (3.2 mmol/L) lower to 42.0 mg/dL (2.3 mmol/L) higher; P=0.754)
³For each additional kg bodyweight, screening blood glucose concentration was estimated to be 7.1 mg/dL (0.4 mmol/L) lower (95% confidence interval 31.4 mg/dL (1.7 mmol/L) lower to 17.3 mg/dL (1.0 mmol/L) higher; P=0.570)
⁴Overall P-value for behaviour score
⁵For each 18 mg/dL (1 mmol/L) increase in fasting blood glucose concentration, screening blood glucose concentration was estimated to be 11.8 mg/dL (0.7 mmol/L) higher (95% confidence interval 13.3 mg/dL (0.7 mmol/L) lower to 37.0 mg/dL (2.1 mmol/L) higher; P=0.355)
⁶After adjusting only for breed, body condition score and behaviour score, for each 18 mg/dL (1 mmol/L) increase in fasting blood glucose concentration, screening blood glucose concentration was estimated to be 10.1 mg/dL (0.6 mmol/L) higher (95% confidence interval 7.4 mg/dL (0.4 mmol/L) lower to 27.6 mg/dL (1.5 mmol/L) higher; P=0.259)
For each 1 g increase in carbohydrate intake, screening blood glucose concentration was estimated to be 0.2 mg/dL (0.0 mmol/L) lower (95% confidence interval 4.8 mg/dL (0.3 mmol/L) lower to 4.4 mg/dL (0.2 mmol/L) higher; P=0.925)
**Table 6.3.** Agreement (and comparisons of means) between screening blood glucose concentrations after various changes in methodology for sampling and measurement that could be expected in a busy veterinary practice. Screening blood glucose: pinna or paw sample on entry to the consulting room measured with a portable glucose meter) was compared with traditional sampling (jugular blood sample after history taking and physical examination) and blood glucose measured either directly from the syringe by meter (traditional-meter), or placed in tube with anticoagulant (sodium fluoride/potassium oxalate (FO) and measured with meter (traditional-meter-FO) and at an external laboratory (traditional-lab-FO).

<table>
<thead>
<tr>
<th>Methodologies compared</th>
<th>N</th>
<th>Lin’s concordance correlation coefficient (95% confidence interval)</th>
<th>Pearson’s correlation coefficient (95% confidence interval)</th>
<th>95% limits of agreement mg/dL (mmol/L)¹</th>
<th>Mean ±SD mg/dL (mmol/L)</th>
<th>Difference mg/dL (mmol/L)</th>
<th>95% confidence interval of difference mg/dL (mmol/L) and p-value for difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Screening versus</td>
<td>105</td>
<td>0.54 (0.40 to 0.66)</td>
<td>0.57 (0.43 to 0.69)</td>
<td>-60 to 44 (-3.4 to 2.4)</td>
<td>106 ± 30 (5.9 ± 1.7)</td>
<td>-8 (-0.5)</td>
<td>-13 to -3 (-0.7 to -0.2) P=0.002</td>
</tr>
<tr>
<td>Traditional-meter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Screening versus</td>
<td>50</td>
<td>0.55 (0.33 to 0.71)</td>
<td>0.56 (0.33 to 0.72)</td>
<td>-57 to 53 (-3.2 to 2.9)</td>
<td>112 ± 32 (6.2 ± 1.8)</td>
<td>-2 (-0.1)</td>
<td>-10 to 6 (-0.6 to 0.3) P=0.551</td>
</tr>
<tr>
<td>Traditional-meter-FO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Screening versus</td>
<td>91</td>
<td>0.51 (0.36 to 0.63)</td>
<td>0.58 (0.42 to 0.70)</td>
<td>-42 to 60 (-2.3 to 3.4)</td>
<td>106 ± 32 (5.9 ± 1.8)</td>
<td>9 (0.5)</td>
<td>4 to 15 (0.2 to 0.8) P=0.001</td>
</tr>
<tr>
<td>Traditional-lab-FO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Traditional-meter</td>
<td>49</td>
<td>0.98 (0.96 to 0.99)</td>
<td>0.98 (0.97 to 0.99)</td>
<td>-9 to 12 (-0.5 to 0.7)</td>
<td>115 ± 28 (6.4 ± 1.5)</td>
<td>1 (0.1)</td>
<td>0 to 3 (0.0 to 0.2) P=0.086</td>
</tr>
<tr>
<td>Traditional-meter-FO</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Traditional-meter</td>
<td>86</td>
<td>0.73 (0.66 to 0.80)</td>
<td>0.95 (0.92 to 0.96)</td>
<td>-2 to 36 (-0.1 to 2.0)</td>
<td>114 ± 26 (6.3 ± 1.5)</td>
<td>17 (0.9)</td>
<td>15 to 19 (0.8 to 1.0) P&lt;0.001</td>
</tr>
<tr>
<td>Traditional-lab-FO</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Traditional-meter-FO</td>
<td>48</td>
<td>0.79 (0.70 to 0.85)</td>
<td>0.96 (0.93 to 0.98)</td>
<td>-1 to 30 (0 to 1.6)</td>
<td>112 ± 25 (6.2 ± 1.4)</td>
<td>14 (0.8)</td>
<td>12 to 17 (0.7 to 0.9) P&lt;0.001</td>
</tr>
<tr>
<td>Traditional-lab-FO</td>
<td></td>
<td></td>
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</tbody>
</table>

¹First listed methodology minus second listed methodology
Figures

**Figure 6.1:** Box and whiskers plot showing blood glucose concentrations in screening samples (collected immediately after entry to the consultation room from pinna or pad) from the cat's first study visit, traditional method (jugular venous sample collected after history and taking and physical exam) and after fasting (sampled from pinna or pad after overnight hospitalisation) from 71 cats 8 years of age or older that had all three samples collected. All samples were tested using a portable glucose meter. (Medians are shown as thick horizontal lines; interquartile ranges and 25\textsuperscript{th} and 75\textsuperscript{th} percentiles are shown by boxes; values more than 1.5 interquartile ranges above the 75\textsuperscript{th} percentile or below the 25\textsuperscript{th} percentiles are each shown as diamonds; error bars show ranges for all less extreme values.)
References


Chapter 7: Metabolomic differences between lean and obese neutered senior cats and associations with glucose tolerance

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Short title: Metabolomics in obese cats

Key words: obesity, diabetes, fatty acids; glycolic acid

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Abstract

Type 2 diabetes is the most common form of diabetes in humans and cats, and obesity is a major risk factor. Metabolomic markers of obesity and predictors of diabetes have been identified in humans, but not in cats. The objectives of the study were to identify metabolite changes with obesity in senior neutered cats, and to establish if changed metabolites were associated with abnormal glucose metabolism and diabetes. Blood samples from 39 cats were analysed: 21 lean (BCS 4-5/9) and 18 obese (BCS 8-9/9). Food was withheld 18 - 24 h and a venous blood sample was collected for GC-MS analysis, and biochemical measures associated with abnormal glucose metabolism and diabetes. Metabolomic analysis identified that 9Z-hexadecenoic acid, tetradecenoic acid, glycerol, and glycolic acid were all increased (P<0.05). Alanine was decreased (P=0.05) and phenylalanine tended to be increased (P=0.09) in obese senior cats. Obesity was characterised by normal screening and fasting blood glucose concentrations, but increased 2 h blood glucose in a glucose tolerance test (GTT) (p<0.01), decreased glucose:insulin ratio (P<0.05), increased plasma leptin (P<0.001), decreased adiponectin (P<0.01), and increased triglyceride (P<0.01) concentrations. There was no significant effect of BCS on FPLi or MCP-1 concentrations. 9Z-hexadecenoic was positively and alanine negatively correlated (P<0.05) with leptin concentrations, and glycolic acid and glycerol were positively associated (P<0.05) with insulin concentrations. This study is the first to report on metabolomic markers of obesity in senior neutered cats, and the identified metabolites need further investigation to determine if they are useful for diagnosing prediabetes in this species.
Introduction

In humans and cats, obesity is often associated with a state of chronic, low grade inflammation, with responses originating primarily in adipose tissue. This inflammation plays a role in the link between obesity and disease, but the disease mechanisms in complex metabolic systems have not yet been fully determined. Inflammatory signals associated with obesity contribute to metabolic dysfunction of carbohydrate, lipid and protein metabolism.

In adipose tissue, inflammatory mediators cause adipocyte insulin resistance and changes in adipocyte gene expression, including decreased adiponectin concentrations which in turn can lead to systemic insulin resistance.

Predisposing factors for diabetes in cats, like humans, are age, obesity and physical inactivity and further in cats, neutering and male gender and breed increase risk. Obese cats are 3.9 times as likely to develop diabetes mellitus than lean cats of matched ages. Clinical signs of diabetes in cats are subtle and progressive over months to years. A major confounding influence on diagnosis in cats are stress effects on blood glucose concentrations at the time of blood sampling (stress hyperglycaemia). Diagnosis of diabetes in cats is usually only be made late in disease progression when overt clinical signs are evident, such as a persistent hyperglycaemia and glucosuria. Approximately 80% of cats with newly-diagnosed diabetes have clinical characteristics and abnormalities consistent with human type 2 diabetes. The typical onset for diabetes in cats and humans appears to be in middle age or later, with over 50% of diabetic cats 10 years or older. As in humans, obesity in cats is associated with increased mortality.

Type 2 diabetes in humans occurs as the result of the failure of multiple organ systems: impaired insulin action in muscles and adipose, poor control of gluconeogenesis by the liver, and insulin deficiency as a result of dysfunction of beta-cells in the endocrine
pancreas\textsuperscript{34-37}. Similar disease characteristics have been found in cats\textsuperscript{11,26,38-40}. A better understanding of the pathophysiological events that contribute to the development of the disease is the key to early diagnosis.

As alterations in metabolic profiles often present much earlier in the course of disease than histopathological changes, metabolomics can be used as a sensitive, early indicator of a disease process, potentially providing a powerful investigative tool for investigating diseases such as diabetes\textsuperscript{28,41}. Candidate biomarkers for insulin resistance have been identified in mice\textsuperscript{28}, and numerous studies have identified the metabolite profiles of insulin resistance and diabetes in humans. These include alterations in essential amino acid metabolism that mark the obese, insulin resistant phenotype\textsuperscript{29-31}. Branched chain amino acids and related metabolites are more strongly associated with insulin resistance than lipids\textsuperscript{7,30}. For example, isoleucine, leucine, valine, tyrosine, and phenylalanine are together associated with a greater than fivefold higher risk of diabetes\textsuperscript{42}, and have been shown to be predictive of development of diabetes in adults up to 12 years in advance, well before alterations in insulin action is detectable by gold standard methods\textsuperscript{31} of insulin sensitivity testing.

There is scant information on metabolomic changes with obesity in animals, despite obesity being common in dogs and cats, as humans. Increased pyruvate and mannose, some long chain fatty acids and lysophospholipids, homocysteine, cysteine, cystine and decreased citrate \(\alpha\)-ketoglutarate and anhydrogluticol have been reported to be associated with weight gain in dogs\textsuperscript{43}, but these changes have not been reported in cats.

Impaired glucose tolerance and impaired fasting glucose are used to diagnose prediabetes in humans, which is an intermediate stage between normal glucose metabolism and clinical diabetes\textsuperscript{44}. Obese humans are four times more likely to have impaired glucose tolerance than normal-weight humans\textsuperscript{45}. We have recently established clinically practical screening tests for
IFG and IGT in high risk cats aged 8 years and older, and found the no obese cats had IFG but 18% had IGT. Furthermore, changes in adipokines (adiponectin and leptin), fPLi, triglycerides and inflammatory mediators have all been associated with obesity and abnormal glucose metabolism in cats, suggesting a role in the development of diabetes. Monocyte chemoattractant protein-1, a chemokine first identified in monocytes, has been found to be significantly increased in humans with obesity and type 2 diabetes but no such reports have yet been made in cats. However, MCP-1 mRNA was detected in subcutaneous and visceral fat, but not in skeletal muscle in cats and the expression of MCP-1 was quantified in isolated islets of healthy cats using feline-specific real-time PCR primers nested within the mRNA sequences.

The primary purpose of this study in cats was to identify metabolic profiles induced by spontaneous obesity in senior animals. Secondly, we determined associations between identified metabolites and measures of abnormal glucose metabolism and diabetes, including insulin, triglyceride, leptin, adiponectin, fPLi and MCP-1 and our glucose screening test based on screening glucose, fasting glucose, and the 2-hour glucose concentration following a simplified glucose tolerance test.
Materials and Methods

The protocol for these studies and the care and handling of these animals were approved by the Animal Experimentation Ethics Committee of the University of Queensland.

Animals: Client-owned cats ≥8 years (n=91) were recruited through veterinary clinics, advertisements and radio interviews between May 2011 and November 2012. Other than for excess body condition in some cats, cats enrolled in the study were assessed as healthy based on results of a clinical examination, patient history, haematological and biochemical assays, total thyroxine, and fPLi. Of the remaining 79 cats, 60 were classified as non-Burmese. Of these cats, samples from 39 cats were used in this study of metabolomics, selected on the basis of client availability/compliance and GC-MS analysis success: 21 lean (BCS 4-5/9; 13 domestic and 8 other breeds; mean age 11.4 years, range 8-18 years; 9 males, 12 females) and 18 obese (BCS 8-9/9; 15 domestic and 3 other breeds; mean age 10.6 years, range 8-16 years; 8 males, 10 females). Additionally, 12 overweight (BCS 6-7/9) cats were used in the analyses of various measures associated with abnormal glucose metabolism and diabetes. All cats used in this study non-Burmese. Incomplete diet histories were available for cats; reliable diet data was only available for 9 of 21 lean and 5 of 18 obese cats. Dietary protein content as a percentage of fed metabolizable energy in lean cats was 24 % ME (all of these cats n=9 were housed together and fed the same diet) whilst in obese cats (n=5) ranged between 24% to 35% (mean ± SEM 27.5 ± 2.3 %). Dietary fat in lean cats was 34% and ranged from 9% to 44% in obese cats (mean ± SEM 27.2 ± 6.5 %). Dietary carbohydrate in lean cats was 36% and ranged from 27% to 45% in obese cats (mean ± SEM 34.0 ± 3.5 %).

Protocol:
On admission, a 5 ml venous blood sample was collected for haematological, biochemical, fPLi and T4 analyses. Fasting blood glucose was measured from a paw or ear sample using a portable glucose meter calibrated for feline blood \(^a\) then an IVGTT was performed using a glucose dose of 0.5 g/kg\(^4\). Samples were analysed by a commercial veterinary laboratory\(^b\).

After food was withheld for 18 to 24 h\(^4\) a venous blood sample (2 mL) was collected for metabolomic assays. Immediately after collection, the blood was placed into clotted red top tubes. Approximately 20 minutes from collection, all samples were centrifuged (8 minutes at 1500 g), serum was separated and stored at -80°C prior to transport on dry ice for GC-MS metabolomic analysis.

**GC-MS Metabolite analysis:**

Serum was assessed for metabolites using gas chromatography- mass spectrometry. A serum sample of 50 µL was transferred into a 1.5 mL Eppendorf® tube. A mix of 150 µL of cold methanol (100 %, 4°C) with 1 µL of a quantitative internal standard\(^g\) added to each sample. The serum mixture was then vortexed for 30 s and then placed on ice for 10 min to precipitate protein. The extracted serum samples were then centrifuged at 13,000 rpm for 10 min at room temperature (23°C). A (30 µL) aliquot of the supernatant was transferred into a glass insert and dried in vacuo for subsequent polar metabolite derivatisation.

The extracted serum samples were re-dissolved in 10 µL of 30 mg mL\(^{-1}\) methoxyamine hydrochloride in pyridine and derivatised at 37°C for 120 min with mixing at 500 rpm. The serum samples were then treated for 30 min with 20 µL \(N,O\)-bis-(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA + 1% TMCS) and the addition of a retention time standard mixture\(^b\) (2.0 µL) with mixing at 500 rpm. Each derivatised sample was allowed to rest for 60 min prior to injection.
Samples (1 μL) were injected using a hot needle technique into a GC-MS system. The MS was adjusted according to the manufacturer’s recommendations using tris-(perfluorobutyl)-amine (CF43). The GC was performed on a 30 m VF-5MS column with 0.2 μm film thickness and a 10 m Integra guard column. The injection temperature was set at 250°C, the MS transfer line at 280°C, the ion source adjusted to 250°C and the quadrupole at 150°C. Helium was used as the carrier gas at a flow rate of 1.0 mL min⁻¹. For the polar metabolite analysis, the following temperature program was used; start at injection 35°C, a hold for 2 min, followed by a 15°C min⁻¹ oven temperature, ramp to 325°C and a final 3 min heating at 325°C. Both chromatograms and mass spectra were evaluated using specialist software. Mass spectra of eluting compounds were identified using a public domain mass spectra library and the in-house Metabolomics Australia mass spectral library. All matching mass spectra were additionally verified by determination of the retention time with authentic standard substances. Resulting relative response ratios (area of analyte divided by area of internal standard, ¹³C₆-sorbitol) per volume of serum for each metabolite was carried out. If a specific metabolite had multiple TMS derivatives, the metabolite with the greater detector response and improved peak shape within the dynamic range of the instrument was selected.

Details of the instrumentation, instrument setting and chromatographic conditions are included in Appendix 7.1.

Hormones and other analytes:
Plasma leptin concentration was measured using a commercially available radioimmunoassay kit, which has been validated for the detection of feline leptin. Intra-assay coefficients of variation (CVs) were 12.3% and 3.9% at mean serum leptin concentrations of 2.3 ng/mL and
11.4 ng/mL, respectively, and the inter-assay CVs were 11.3% and 6.3% at mean leptin concentrations of 2.6 ng/mL and 11.4 ng/mL, respectively.

Serum total adiponectin concentration was determined by a commercially available murine/rat adiponectin ELISA kit. The assay was validated for the detection of feline adiponectin. Intra-assay CV was 3.2% and 3.7% for adiponectin concentrations of 21.7 µg/mL and 4.2 µg/mL, respectively.

Feline pancreatic lipase concentrations were determined at a commercial laboratory by radioimmunoassay validated for use in the cat. The intra-assay coefficients of variation of 4 different serum samples ranged from 2.2 to 10.1%.

Serum triglyceride concentrations were determined using an autoanalyser (Hitachi-7180) using manufacturer’s reagents. The intra-assay coefficient of variation was 2.2% and the inter-assay coefficient was 2.2 and 5.7%.

Serum insulin concentrations were measured at a research laboratory using a commercial ELISA kit validated for feline insulin. Intra- and inter-assay reproducibility of feline insulin ELISA kit are less than 10%.

MCP-1 concentrations were determined at a research laboratory using the canine MCP-1 ELISA kit. This was validated by assaying two feline serum samples: MCP-1 concentrations were 163.8 and 316.9 pg/ml, CV were 3.8% and 3.5% (intra-assay), correlation coefficients of the diluted samples were 0.998 and 1.00, respectively. Recovery of the applied recombinant MCP-1 at varied concentrations ranged from 92% through 100%.
Statistical analyses:

As metabolites in a data matrix are often heteroscedastic and have a right skewed distribution, the data were log transformed. Using the metabolomics package of R⁶, metabolite data were normalized using a single internal standard method where a normalized data matrix is obtained by subtracting the log metabolite abundance of a single internal standard from the log abundances of the metabolites in each sample⁶⁶. The normalized data was visualized using a RLA plot⁶⁶ where the median of each metabolite in the data matrix, and then subtracted this median from each metabolite. Ideally the medians would be as close to zero as possible, but this may vary if there are numerous significantly abundant metabolites. Principal component analysis plots of the normalized log transformed data were used to aid in visualizing possible variation, clustering tendencies, trends and outliers.

A linear regression model (adjusting for the covariances of body condition, sex and age) was used to identify significant metabolites and fold changes were calculated to rank the metabolites of significance in the pairwise comparisons. P-values are presented as unadjusted, and adjusted using the Benjamini-Hochberg step-up false discovery rate method⁶⁸. Adjusted P-values <0.05 were considered significant and <0.1 were considered trending towards significance.

The effect of BCS on measures associated with abnormal glucose metabolism and diabetes (screening, fasting and 2-h blood glucose following a glucose tolerance test, leptin, adiponectin, L:A ratio, fPLi, Insulin, G:I ratio, triglyceride, and MCP-1) was analysed by one-way ANOVA. A post-hoc Tukey’s multiple comparison test was used to determine significant differences between lean, overweight and obese cats. Pearson correlations were used to examine relationships between significant metabolites identified from metabolomics.
analysis and measures associated with abnormal glucose metabolism and diabetes for lean and obese cats.
Results

Metabolic characterization of cats

Glucose variables for both lean and obese cats have been reported previously\textsuperscript{46,47}. Fasting blood glucose concentrations (mean ± SEM) were not different between lean and obese cats (5.2 ± 0.4 mmol/L lean; 4.6 ± 0.2 mmol/L obese cats). Glucose concentrations 2 hr following a GTT were significantly higher (P<0.01) in obese cats (5.8 ± 0.3 mmol/L lean; 7.9 ± 0.6 mmol/L obese), and 18% (3/17) obese cats but no lean cats had impaired glucose tolerance (glucose > 9.8 mmol/L at 2 hr). Mean fasting insulin concentrations were 50% higher in obese cats (0.6 ± 0.1 ng/ml lean; 0.9 ± 0.2 ng/ml obese) but the difference was not statistically significant (P=0.1). Mean fasting glucose:insulin ratio in obese cats was only 1/3\textsuperscript{rd} of lean cats (22.0 ± 8.2 lean; 7.5 ± 1.1 obese; P <0.05). Triglyceride concentrations were 2.5 times higher in obese than lean cats (18.9 ± 2.4 lean; 54.2 ± 19.1 obese, P< 0.01) (Table 7.1). Leptin concentrations were higher (P<0.05), whilst adiponectin concentrations were lower (P<0.05) than lean cats, but MCP -1 was not different between lean and obese cats (Table 7.1).

Metabolomic differences between lean and obese cats

GC-MS analyses identified 173 metabolites (55 identified by the metabolomics libraries and 118 unknown) in this study of lean and obese senior cats. Relative log abundance (RLA) plot across all cats exhibited medians close to zero and acceptable variation (Figure 7.1). Principal component analysis (PCA) plots identified distinct clustering of metabolites between the lean and obese cats (Figure 7.2). Thirteen metabolites were found to be significantly different between lean and obese cats, and of these 6 metabolites were identified (Table 7.2). Identified non-significant metabolites are listed in Appendix 7.2. Obese cats exhibited a 1.8 to 2.0-fold
increase (P<0.05) in the fatty acids; tetradecenoic (myristoleic) and 9Z-hexadecenoic (palmitoleic) acid, together with a 1.4-fold increase (P<0.05) in glycerol. Furthermore, obese cats had decreased alanine and increased phenylalanine, although both of these amino acids were only approaching significance (corrected P=0.052 and P=0.093, respectively). Other amino acids; leucine, isoleucine, valine, tyrosine and tryptophan were not significantly different between lean and obese cats. Glycolic acid was increased 1.4-fold (P<0.05) in obese cats.

Correlations between significant metabolites and measures of abnormal glucose metabolism and diabetes

Moderate significant correlations were found between metabolites identified by GC-MS analysis and measures of abnormal glucose metabolism and diabetes (Table 7.3). Alanine was negatively correlated with 2-h blood glucose and leptin. Phenylalanine was negatively correlated with fPLi and positively correlated with insulin, but not correlated with blood glucose measures. 9Z-hexadecenoic acid was positively correlated with leptin, whilst tetradecenoic acid was negatively correlated with glucose:insulin ratio. Neither fatty acid was significantly correlated with triglyceride or insulin. Glycerol was negatively correlated with fasting blood glucose and glucose:insulin ratio, and positively correlated with insulin. Glycolic acid was negatively associated with adiponectin and positively associated with insulin. No significant correlations were found between identified metabolites and MCP-1.
Discussion

Numerous studies have reported alterations in amino acid$^{34,68}$, and fatty acid metabolism$^{69,70}$ in obese humans and identified several metabolites as predictors of insulin resistance$^{71,72}$ and type 2 diabetes$^{31,73,74}$. In cats there are fewer reports of such predictive markers$^{55,75,76}$. Using a metabolomic approach we examined differences between obese and lean senior neutered cats, and overall identified 6 metabolites that were significantly altered. Obese cats were characterised by altered lipid metabolism with increased triglycerides, tetradecenoic and 9Z-hexadecenoic (palmitoleic) fatty acids, together with increased glycerol. For amino acids, phenylalanine was increased in obese cats, whilst surprisingly alanine was decreased. Also, we identified a novel metabolite glycolic acid (glycolate) as increased in obese cats, suggesting that dysregulation of glycolic acid metabolism might be characteristic of obesity and prediabetes in cats. Additionally, other metabolites measured in this study were altered between our lean and obese study cats, namely leptin and leptin:adiponectin ratio and triglyceride concentrations significantly increased and adiponectin and glucose:insulin ratio significantly decreased in obese cats. Neither insulin nor MCP-1 were not altered between the two groups in our study.

Alterations in fatty acid metabolism

Obese cats are known to exhibit hyperlipidaemia and dyslipidemia$^{40}$ with increased triglycerides, cholesterol, and lipoproteins (VLDL and HDL)$^{74}$. In the present study, obese cats had increased triglycerides (others were not measured). Similarly, in humans, obesity is characterised by dyslipidaemia$^{77}$ with increased FFA, triglycerides and cholesterol, albeit decreased HDL$^{78}$. Increased FFA concentrations in humans are linked to the onset of insulin resistance with increased flux of FFA leading to an overproduction of VLDL$^{3}$ and the
dyslipidaemia is thought to be a crucial link between insulin resistance and beta-cell dysfunction\textsuperscript{79} in type 2 diabetes. In our study obese cats had a significantly lower glucose:insulin ratio that the lean cats.

Two fatty acids, palmitoleic and tetradeconoic acids were increased in obese cats when compared with lean cats. Studies in humans have reported a relationship between palmitoleic acid and obesity\textsuperscript{74,80} and type 2 diabetes\textsuperscript{81-83}, but our results are novel in cats. However, in humans there is controversy over whether palmitoleic acid improves metabolic parameters in obesity and insulin resistance\textsuperscript{84} or increased circulating palmitoleic acid concentrations are associated with increased incidence of diabetes\textsuperscript{81,83-85}. Palmitoleic acid appears to have many complex roles in lipid metabolism, and cannot easily be defined as a predictive marker of insulin sensitivity and diabetes\textsuperscript{86}. In diabetic rodent models, administration of palmitoleic acid has been noted to improve hyperglycaemia and hypertriglyceridemia reduces insulin resistance and hepatic lipid accumulation\textsuperscript{87,88}. Our results showed a positive correlation between palmitoleic acid and leptin, but no association with insulin, triglycerides, or any other measures associated with abnormal glucose metabolism and diabetes. Although in our cats, insulin was not significantly increased with obesity, mean concentrations were nearly double those in lean cats, and fasting glucose:insulin ratio was significantly lower in obese than lean cats (P<0.05). The other fatty acid identified as increased in obese cats was tetradeconoic acid, a saturated fatty acid. Altogether fatty acids have been considered independent predictors of progression to diabetes in human studies\textsuperscript{69,89} by directly impairing insulin actions via oxidative stress, inflammation and mitochondrial dysfunction\textsuperscript{89}. Long chain fatty acids, such as tetradeconoic acid, are considered markers of oxidative stress\textsuperscript{90-92}. Interestingly, there was a negative correlation between myristoleic acid and glucose:insulin ratio in cats, supporting the notion that saturated fatty acids potentiate glucose-induced
insulin secretion which has been reported pancreatic islets extracted from female NMRI mice\textsuperscript{93}. Therefore, myristoleic acid may be a useful indicator of oxidative stress and early beta-cell dysfunction in cats. Glycerol, the “backbone” of triglycerides was also found to be increased in the obese cats. In humans, plasma glycerol is reported to increase in early phase of obesity\textsuperscript{94} and predict a worsening of hyperglycaemia and the development of type 2 diabetes\textsuperscript{95}. Our study identified a positive correlation between glycerol and insulin, and negative correlation between glycerol and both fasting blood glucose and glucose:insulin ratio in cats. Although the negative of glycerol and fasting blood glucose was contrary to results reported in humans, none of the obese cats in our study had increased fasting glucose consistent with IFG and only 18\% of obese cats had IGT. The negative association of glycerol with glucose:insulin ratio and positive association with insulin in our cats are consistent with findings in humans where fasting levels of glycerol are increased with impaired fasting glucose, impaired glucose tolerance and newly diagnosed diabetes\textsuperscript{95}. Although circulating FFA and glycerol levels are significantly increased in humans with obesity, they are only marginally influenced by insulin resistance and type 2 diabetes\textsuperscript{96}, and suggest that glycerol may not be a reliable measure of progression to diabetes in obese patients.

Obesity is characterised by an expansion of white adipose tissue mass and is associated with increased leptin and decreased adiponectin plasma concentrations as the obesity progresses\textsuperscript{5,49}. Our obese cats were also characterized by increased leptin and decreased adiponectin concentrations. The relationship between obesity and adipokines as markers of inflammation has been extensively studied in humans\textsuperscript{97,98}, dogs\textsuperscript{99}, and cats\textsuperscript{5,48,50,100}. With increasing adiposity, there is a general increase in leptin, and the current results agree with previous studies in cats\textsuperscript{63,101,102}. The main physiologic role of leptin is to regulate body fat mass through modulating satiety and energy metabolism, although previously in cats we have
shown that higher plasma leptin concentrations are associated with insulin resistance, independent of the degree of adiposity\textsuperscript{102}. Leptin can act to sensitize the body to insulin by increasing fatty acid oxidation and decreases triglyceride content in the liver and other tissues\textsuperscript{5}. In contrast, adiponectin was decreased in our obese cats, consistent with reports that circulating adiponectin concentrations are decreased with obesity in humans\textsuperscript{103} and cats\textsuperscript{5,104,105}. Adiponectin has insulin-sensitizing effects, increasing fatty acid oxidation and decreasing triglyceride content in the liver and other tissues\textsuperscript{5} and reduced adiponectin in obesity likely contributes to insulin insensitivity.

\textit{Glycolic acid}

Glycolic acid (also known as glycolate) was a novel metabolite identified as increased in our cohort of obese cats. It has recently been shown to be increased in obese humans that display dyslipidaemia and hyperglycaemia, compared to healthy obese people\textsuperscript{106}. Glycolic acid is a metabolite related to mitochondrial and peroxisome function in hepatocytes, and oxalic acid (oxalate) metabolism. Glycolic acid can be metabolised to glyoxylic acid (glyoxylate) by glycolate oxidase in peroxisomes, and then to oxalate by lactate dehydrogenase predominantly in the cytoplasm. Oxalate and glycolate can be excreted in urine, or the oxalate can be retro-converted to glycolate\textsuperscript{107} or glyoxylic acid\textsuperscript{108}. Whilst the underlying cause for an increase in glycolic acid in obese cats and humans remains to be determined, studies have linked increased glyoxylate to prediabetes in humans, it is hypothesized that glyoxylate metabolism contributes to fat-induced hepatic insulin resistance and associated hyperglycemia\textsuperscript{109,110}. The obese cats in our study had reduced glucose tolerance compared to lean cats based on significantly increased 2 hr glucose concentrations in a GTT, and 16\% of obese cats had impaired glucose tolerance and were considered prediabetic. Furthermore, glyoxylate has been identified as a unique marker of prediabetic metabolism, with increased
glyoxylate being detected in subjects up to 3 years prior to diagnosis of diabetes mellitus\textsuperscript{111}. Also in a diabetic animal model (\textit{db} mouse) increased plasma glyoxylate concentrations have been reported, but interestingly glycolic acid concentrations were normal \textsuperscript{109}. Unfortunately, in our study, glyoxylate was not detectable by GC-MS.

An increase in circulating glycolic acid concentrations in obese cats could result in acidosis. In ethylene glycol toxicity in cats and dogs the hepatic metabolism of ethylene glycol leads to an accumulation of glycolic and oxalic acids and this results in a severe, often lethal, metabolic acidosis\textsuperscript{112}. In obese cats in our study, the small (1.4-fold) increase in glycolic acid is likely to produce a mild metabolic acidosis, and this could potentially predispose cats to diabetes. In humans, even mild metabolic acidosis is known to produce insulin resistance in seemingly healthy adults\textsuperscript{113}, and a link between dietary acid load such as a diet rich in animal products, is associated with chronic metabolic acidosis, insulin resistance, metabolic syndrome, and an increased risk of type 2 diabetes\textsuperscript{114}. Similarly, cats being obligate carnivores traditionally, consume a particularly acidotic diet, although this has changed somewhat during domestication with increased levels of carbohydrate in commercial diets\textsuperscript{115}. Obese cats are often converted to prescription diets with higher protein than typical feline diets to promote weight loss\textsuperscript{116-118}. In our obese cats, the range of dietary protein intake was only 24 to 35\% of ME, in those animals with good dietary records. It would therefore appear unlikely that high protein/meat acidotic diets \textit{per se} are the cause of increased glycolic acid in our cohort of obese cats. Rather the increased glycolic acid is likely associated with dysregulation of liver metabolism in obesity and a prediabetic state. In support of this notion, concentrations of glycolic acid are significantly elevated in the urine of streptozotocin-induced diabetic rats, regardless of the type of diet fed\textsuperscript{119}. In this rodent model, the source of increased glycolic acid was unresolved, but was suggested to relate to either decreased rate of
glycolic acid utilization and/or excretion, or increased rate of synthesis. In our study, it should be noted that glycolic acid concentrations in cats were negatively associated with adiponectin and positively associated with insulin. Together these results imply increased glycolic acid is associated with reduced insulin sensitivity. Further work is required to investigate the metabolism of glycolic acid in obesity, and determine whether glycolic acid and other related metabolites (glyoxylate and oxalate) may be useful in diagnosing prediabetes in cats.

Amino acids

Obesity and insulin resistance in humans is associated with altered amino acid metabolism, in particular, high concentrations of branched chain amino acids (BCAA). Increases in leucine, isoleucine and valine, along with alanine (as a by-product of BCAA metabolism), are reported to predict worsening insulin sensitivity in humans. However, in our cohort of obese cats we did not identify any changes in BCAA metabolism, except a trend towards decreased alanine concentrations. Nevertheless, alanine concentrations were negatively correlated with 2-h blood glucose following a GTT, suggesting a link between alanine and glucose intolerance. Further analysis of results for obese cats showed a positive correlation (r=+0.60, p<0.05) between alanine and insulin concentrations, which is in accord with findings that alanine has weak insulinotropic effects in adult cats. Decreased alanine levels in obese cats could be indicative of increased gluconeogenesis (alanine to pyruvate to glucose), and/or increased glyoxylate detoxification in hepatic peroxisomes (glyoxylate can combine with alanine to generate pyruvate). However, neither pyruvate nor glucose (screening or fasting or metabolomics) were significantly increased in the obese cats. Whether alanine is linked to downstream metabolism of glycolic acid in obese cats needs further investigation.
Phenylalanine, an essential α-amino acid, was also increased in obese cats. Increased phenylalanine levels have been described in obese humans and as a marker of insulin resistance. In our cats, phenylalanine had a positive association with insulin concentrations, similar to metabolomic findings in humans at risk for diabetes. However, our data showed no correlation between phenylalanine and fasting glucose, unlike human studies where increased phenylalanine is associated with impaired fasting glucose and type 2 diabetes. This may have been because none of our cohort of obese cats had impaired fasting glucose. Phenylalanine is a glucogenic amino acid and as such is deaminated in the liver to produce glucose. Interestingly the natural diets of cats are essentially carbohydrate-free and in general, cat metabolism is directed towards deriving energy more from dietary proteins and fats. Deng et al. studied the diets of cats and identified increased phenylalanine as a marker of high protein diets (50% ME). In contrast, Silva and Mercer suggested that the amount of amino acid gluconeogenic precursors was unaffected in isolated feline hepatocytes by dietary protein intake when comparing diets containing 18% and 70% ME protein. In our study, the diets of the cats were variable and they were frequently fed a combination of diets, as cats were client owned. Further controlled dietary studies with obese cats are needed to validate our results.

MCP-1
Surprisingly in our study, MCP-1 was not significantly increased in obese cats. This is contrary to findings in humans, where increased MCP-1 concentrations occur with obesity and are associated with type 2 diabetes, in dogs with obesity, and in mice, MCP-1 induces inflammation in adipose tissue. In cats, MCP-1 mRNA has been detected in subcutaneous and visceral fat but not in skeletal muscle, and in humans, MCP-1 protein expression was higher in omental fat than in subcutaneous fat in severely obese patients.
One possible explanation for the lack of MCP-1 expression is the distribution of fat in obese domestic cats. In contrast to humans, obese cats had abdominal fat equally distributed subcutaneously and intra-abdominally\textsuperscript{137}. Further studies are needed to determine whether MCP-1 plays a role in the development of diabetes in cats.

**Limitations**

In this study, there are a few noteworthy limitations. Firstly, our cats were all client-owned cats, not research cats, so the number of subjects is small and a period of acclimatization was not possible. It was assumed that the cats were spontaneously obese, and given all cats were aged (≥8 years), it is presumed that they had been obese for several years. However, no data were obtained on the duration of obesity. Secondly, the lack of complete diet records for all animals did not enable analysis of the metabolomics results in relation to potential differences in dietary composition, although no major differences in diet were noted between animals with good records. Further, as the animals were client-owned cats we were unable to normalise diets for all animals prior to metabolomics analysis. Thirdly, 7 of the metabolites that were significantly different between lean and obese cats could not be identified, and would require additional laboratory-based analyses to identify them. In addition, there is a limitation of using GC-MS in detecting non-volatile compounds, and these would have been better determined using LC-MS. A targeted metabolomic approach in a longitudinal study would be useful to determine if changes in metabolites predict the development clinical diabetes in the future. As Ferrer et al\textsuperscript{138} stated, ‘being obese is not healthy and is even less so if insulin resistance and diabetes ensues’, and thus indicators of metabolic derangements of obesity and predictors of future diabetes are urgently needed.
Conclusion

We have identified metabolic profiles induced by spontaneous obesity in senior cats. The results indicate specific alterations in lipid and amino acid profiles, together altered glycolic acid metabolism. These metabolites might prove useful markers of metabolic dysfunction in obesity, and improve understanding of the metabolic derangements leading to diabetes in cats.

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Footnotes

a AlphaTRAK (Zoetis)

b Idexx Laboratories, Brisbane Australia
c Linco's Multi-Species Leptin Radioimmunoassay (RIA) Kit, Linco Research Inc, St Charles, MO

d B-Bridge international, Otsuka, Tokyo, Japan

e Nippon University

f Morinaga Institute of Biological Science, Kanagawa, Japan

g $^{13}$C$_6$-Sorbitol/$^{13}$C$_5^{15}$N-Valine in methanol, 0.5 mg mL$^{-1}$

h 0.029% (v/v) $n$ dodecane, $n$-pentadecane, $n$-nonadecane, $n$-docosane, $n$-octacosane, $n$-dotriacontane, $n$-hexatriacontane dissolved in pyridine

i Gerstel 2.5.2 autosampler, a 7890A Agilent gas chromatograph and a 5975C Agilent quadrupole MS (Agilent, Santa Clara, USA).

j Varian, Inc, Victoria, Australia

k Agilent MassHunter Workstation Software, Quantitative Analysis, Version B.05.00/Build 5.0.291.0 for GCMS

l Max-Planck-Institute for Plant Physiology, Golm, Germany (http://csbdb.mpimp-golm.mpg.de/csbdb/dbma/msri.html)

m ($^{13}$C$_6$H$_4$O$_6$ – Sorbitol - (0.5 mg/mL), $^{13}$C$_5^{15}$N-Valine - (0.5 mg/mL), 2-aminoanthracene - (0.25 mg/mL) Pentafluorobenzoic acid - (0.25 mg/mL))


o GraphPad Prism version 7.01. GraphPad Software, Inc. La Jolla, CA, USA.

Reference Interval Draft Version, Copyright 2005, University of Cincinnati
Tables

Table 7.1. Measures associated with abnormal glucose metabolism and diabetes for lean, overweight and obese senior cats. Values are mean ± SEM. Different superscripts denote significant (P<0.05) difference between groups, as determined by post-hoc Tukey multiple comparison test.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Lean</th>
<th>Overweight</th>
<th>Obese</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BCS 4-5/9, n=18-19</td>
<td>BCS 6-7/9, n=12</td>
<td>BCS 8-9/9, n=17</td>
<td></td>
</tr>
<tr>
<td>Screening Blood Glucose (mmol/L)</td>
<td>6.0 ± 0.5</td>
<td>5.4 ± 0.5</td>
<td>6.4 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td>Fasting Blood Glucose (mmol/L)</td>
<td>5.2 ± 0.4</td>
<td>4.9 ± 0.2</td>
<td>4.6 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>2-hr Blood Glucose (mmol/L)</td>
<td>5.8 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.4 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.9 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>11.1 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.4 ± 3.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39.6 ± 5.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Adiponectin (µg/mL)</td>
<td>6.6 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.1 ± 0.9&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.5 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Leptin:Adiponectin ratio (ng/µg)</td>
<td>3.9 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.4 ± 3.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.2 ± 12.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>fPLi (µg/L)</td>
<td>1.7 ± 0.3</td>
<td>2.7 ± 1.3</td>
<td>2.0 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td>Insulin (ng/mL)</td>
<td>0.6 ± 0.1</td>
<td>1.0 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>Fasting glucose:insulin ratio</td>
<td>22.0 ± 8.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.5 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.5 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>18.9 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.1 ± 6.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54.2 ± 19.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MCP-1 (pg/mL)</td>
<td>64.2 ± 5.3</td>
<td>83.6 ± 19.0</td>
<td>64.6 ± 13.5</td>
<td>NS</td>
</tr>
</tbody>
</table>

**fPLi** feline pancreatic lipase

**MCP-1** Monocyte chemoattractant protein-1

**BCS** body condition score

**NS** not significant (P≥0.05)

a,b,c Significant differences between lean, overweight and obese cats
Table 7.2 Significant identified metabolites from GC-MS analysis of lean and obese senior cats. Values are mean ± SEM expressed as fold-change relative to mean for lean (control) group. P-values from pair-wise unpaired t-test comparisons are presented as unadjusted, and adjusted using the Benjamini-Hochberg step-up false discovery rate method.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Lean Cats n = 21</th>
<th>Obese Cats n = 15</th>
<th>Unadjusted P-value</th>
<th>Adjusted P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>1.0 ± 0.08</td>
<td>0.6 ± 0.12</td>
<td>0.01</td>
<td>0.09</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.0 ± 0.08</td>
<td>1.6 ± 0.08</td>
<td>0.001</td>
<td>0.05</td>
</tr>
<tr>
<td>Tetradecanoic acid</td>
<td>1.0 ± 0.11</td>
<td>1.8 ± 0.09</td>
<td>0.001</td>
<td>0.03</td>
</tr>
<tr>
<td>9Z- hexadecenoic acid</td>
<td>1.0 ± 0.10</td>
<td>2.0 ± 0.11</td>
<td>0.002</td>
<td>0.04</td>
</tr>
<tr>
<td>Glycerol</td>
<td>1.0 ± 0.09</td>
<td>1.4 ± 0.05</td>
<td>0.001</td>
<td>0.03</td>
</tr>
<tr>
<td>Glycolic acid</td>
<td>1.0 ± 0.08</td>
<td>1.4 ± 0.06</td>
<td>0.001</td>
<td>0.03</td>
</tr>
</tbody>
</table>
Table 7.3. Pearson correlation of metabolites identified from GC-MS analysis of lean and obese senior cats and measures associated with abnormal glucose metabolism and diabetes (n=36).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Measure of glucose metabolism</th>
<th>r</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amino Acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>2-hr blood glucose</td>
<td>-0.45</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>Leptin</td>
<td>-0.58</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>fPLi</td>
<td>-0.34</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Insulin</td>
<td>+0.50</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Fatty Acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9Z-hexadecenoic acid</td>
<td>Leptin</td>
<td>+0.36</td>
<td>0.03</td>
</tr>
<tr>
<td>Tetradecenoic Acid</td>
<td>G:I ratio</td>
<td>-0.35</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Glycerol</strong></td>
<td>Fasting blood glucose</td>
<td>-0.33</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Insulin</td>
<td>+0.35</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>G:I ratio</td>
<td>-0.35</td>
<td>0.03</td>
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<tr>
<td><strong>Other acids</strong></td>
<td></td>
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<tr>
<td>Glycolic acid</td>
<td>Adiponectin</td>
<td>-0.45</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>Insulin</td>
<td>+0.37</td>
<td>0.03</td>
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Figures
Figure 7.1: A relative log abundance (RLA) plot where the median of each metabolite in the data matrix is visualized, and then subtracted this median from each metabolite. Blue bars are lean (BCS 4-5/9) and green bars are obese (8-9/9) non-Burmese cats. Red, purple and yellow bars are related to chapter 8.
**Figure 7.2:** Principal Component Analysis (PCA) plot of GC-MS data showing distinct clustering of metabolic profiles between lean (red) and obese (blue) senior cats discriminated based on the thirteen significant metabolites.
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Chapter 8: Metabolomic differences between healthy senior Burmese and non-Burmese cats

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Short title: Diabetic markers in Burmese cats

Key words: diabetes; insulin, glucogenic amino acids, dyslipidaemia

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Abstract

Burmese cats exhibit dyslipidaemia and are 4 times more likely to develop diabetes than other cat breeds. The aim of this study was to identify metabolomic differences between healthy senior Burmese (n=19) and non-Burmese (n=30) cats, and to examine correlations between significant metabolites and measures known to be associated with abnormal glucose metabolism and diabetes. Food was withheld 18 - 24 hr and a venous blood sample collected for GC-MS analysis, biochemical and hormonal assays, and a glucose tolerance test was performed. Metabolomic analysis (corrected for age, gender and body condition score) identified 23 known metabolites significantly (P<0.05) different between Burmese and non-Burmese cats. Many glucogenic amino acids, together with hydroxyproline and aminobutyric acids were all increased, whilst beta-alanine was decreased in Burmese cats. Fructose and glucose, cholesterol and some fatty acids, glycerol-3-phosphate, numerous acids (lactic acid, glycolic acid, phosphoric acid, threonic acid, glycerate, and succinate) were all decreased in Burmese cats. Urea was increased in Burmese. Biochemical testing revealed that Burmese cats exhibited increased (P<0.05) insulin, triglyceride, and MCP-1 concentrations. Positive moderate associations were found between 7 amino acids (beta-alanine, cysteine, glutamine, hydroxyproline, phenylalanine, proline and tyrosine) identified by metabolomics and insulin. No differences were observed in plasma adipokines. This study is the first to report metabolite differences between healthy non-Burmese and Burmese cats. The results highlight potential markers of metabolic dysfunction in a cat breed at high risk of developing diabetes.
**Introduction**

Burmese cats from the UK, Sweden, Australia and New Zealand are four times more likely to develop diabetes mellitus than non-Burmese\textsuperscript{1-4}, with one in 50 Burmese in the UK and Australia being affected\textsuperscript{2,5}. Diabetes in Burmese closely resembles type 2 diabetes in humans, based on common pathophysiologic features. These include late age of onset, insulin resistance\textsuperscript{6-11}, loss of beta-cell mass\textsuperscript{12-15}, and amyloid deposition in pancreatic islets\textsuperscript{16-18}.

Burmese cats in New Zealand have evidence of an inherited component, involving a major locus with a significant risk allele to diabetes\textsuperscript{3}. Formation of the breed occurred in 1957 from only a very small number of individuals\textsuperscript{20,21}, and males and females are similarly affected, indicating that the gene or genes causing diabetes are autosomal rather than sex-linked\textsuperscript{3}. A gene expression study reported that lean Burmese displayed a 3- to 4-fold increase in the very low density lipoprotein percentage, double that for obese domestic cats, suggestive of lipid (particularly triglyceride) dysregulation\textsuperscript{22}. Approximately, 28% Australian Burmese cats have altered lipid metabolism, with an exaggerated postprandial triglyceride response after an oral fat tolerance test, indicating possible insulin dysregulation\textsuperscript{10,23}. However, affected Burmese cats have normal blood fasting insulin, fructosamine, non-esterified free fatty acids, and apolipoprotein concentrations\textsuperscript{23}.

In Burmese cats, fasting blood glucose concentrations and glucose tolerance test results are not different to non-Burmese cats, even when results are adjusted for body condition score, age and gender\textsuperscript{24,25}. Therefore, other markers of altered metabolism in Burmese cats need to be investigated to identify differences between Burmese and non-Burmese cats that might account for their increased risk of developing diabetes.
Metabolomics involves measurement of a comprehensive set of metabolites in a body fluid\textsuperscript{26}. Alterations in metabolic profiles are often present much earlier in the course of disease than histopathological changes, and can therefore be used as a sensitive, early indicator of a disease process\textsuperscript{27}. We found metabolomic differences between lean and obese senior non-Burmese cats (\geq 8 years of age) (Chapter 7), including changes in lipids, amino acids, and glycolic acid metabolism. It is unknown whether the metabolomic characteristics of obese non-Burmese cat also occur in senior Burmese cats. Obesity is a risk factor for type 2 diabetes in both domestic and Burmese cats\textsuperscript{28}, and obesity in domestic cats is associated with abnormal glucose metabolism, and changes in circulating levels of adipokines (adiponectin and leptin)\textsuperscript{29-33}, fPLI\textsuperscript{34}, triglycerides\textsuperscript{22,35} and inflammatory disease mediators\textsuperscript{36} but not with MCP-1 (Chapter 7). However, many of these markers have not been examined in Burmese cats, and apart from altered lipid metabolism, it is generally unknown if there are other characteristic differences in the Burmese metabolome\textsuperscript{22}.

Therefore, the aims of this study were to compare metabolomics profiles and some other indicators of obesity between healthy senior Burmese and non-Burmese cats, adjusted for differences in body condition, sex and age. In addition, we determined whether identified metabolites were associated with abnormal measures of glucose metabolism, including screening\textsuperscript{24}, fasting and the 2-h glucose concentrations\textsuperscript{25} following a glucose tolerance test.
Materials and Methods

The protocol for these studies and the care and handling of these animals were approved by the Animal Experimentation Ethics Committee of the University of Queensland.

Animals:

Client-owned cats (n=49) were recruited for this study and were presented for a routine health check at local veterinary clinics in Brisbane. Each cat was assessed and classified as either Burmese (n=19) or non-Burmese (n=30). All the Burmese cats had a known history of being acquired from breeders. Other than for excess body condition in some cats, cats enrolled in the study were assessed as healthy based on results of a clinical examination, patient history, haematological and biochemical assays, total thyroxine, and fPLi. Body condition score (BCS) was recorded on scale of 1 to 9, and cats between 4 and 9 were included. Body condition scores for Burmese were 6 lean (BCS 4-5), 11 overweight (BCS 6-7) and 2 obese (BCS 8-9) with overall mean 6.1, range 4-8, and for non-Burmese (all domestic cats) were 12 lean, 5 overweight and 13 obese, with overall mean 6.1 and range 4-9. Ages of the cats ranged from 8 to 15 years (mean ± SEM 9.9 ± 0.39 years) for Burmese and 8 to 18 years (mean ± SEM 10.7 ± 0.34 years) for non-Burmese. The Burmese consisted of 8 neutered females and 11 neutered males, and the non-Burmese had 16 neutered females and 14 neutered males. Incomplete diet histories were available: 6/19 (32%) of the Burmese and 16/30 (53%) of the non-Burmese cats in our study had reliable diet information. Dietary protein as a percentage of metabolizable energy was 20% to 31% ME in Burmese versus 24% to 35% in non-Burmese (mean ± SEM 26.5 ± 1.7 and 25.1 ± 0.3, respectively). Dietary fat in Burmese was 34% to 40% and non-Burmese 15% to 44% (mean ± SEM 37 ± 1.0 and 33.4 ± 1.6, respectively). Dietary carbohydrate in Burmese was from 34% to 39% and in non-Burmese 26% to 36% (mean ± SEM 36.5 ± 0.8 and 34.6 ± 0.9, respectively). P-values using an unpaired t-test did not show significant differences (P<0.05) between the subgroups.
Protocol:

On admission after a full physical examination, a 5-ml venous blood sample was collected for standard haematology, biochemical profile, feline pancreatic lipase, and total thyroxine analyses to determine if animals were suitable for inclusion in the study. Samples were analysed by a commercial veterinary pathology laboratory.

After examination, cats were hospitalized and food was withheld for 18 to 24 hr and jugular venous blood samples were collected for hormone (4 mL EDTA plasma and serum) and metabolomic (2 mL serum) analyses. Blood for serum was allowed to clot for 20 minutes., All samples were centrifuged for 8 minutes at 1500 g then plasma/serum separated and stored at -80°C until transport on dry ice and analysis. To allow for resolution of stress hyperglycaemia, fasting blood glucose was measured from a paw or ear sample using a portable glucose meter calibrated for feline blood 3 h after catheter placement and then an IV glucose tolerance test performed using a glucose dose of 0.5 g/kg. A timer was started halfway through the infusion and blood samples were taken at 2 min, 2 h and then hourly until glucose returned to below our laboratory’s upper limit of normal fasting glucose concentration of 6.5 mmol/L.

Gas chromatography- mass spectrometry metabolomic analysis:

Serum was assessed for metabolites using gas chromatography/mass spectrometry. Detailed methods are described elsewhere (Chapter 7). Briefly, extraction was performed using cold methanol and internal standard added. The sample was then placed on ice to precipitate the protein, centrifuged, dried and then redissolved for polar derivatisation. Samples were injected
using a hot needle technique into a GC-MS system. Chromatograms and mass spectra were evaluated using specialist software and metabolites identified using a library.

**Hormones and other analytes:**

Plasma leptin and serum total adiponectin concentrations were measured using commercially available radioimmunoassay and ELISA kits respectively, which have both been previously validated for the detection of feline adipokines. Intra-assay and inter-assay coefficients of variation (CVs) were <15% and <10% for leptin quality controls of 2.3 ng/mL and 11.4 ng/mL respectively. For adiponectin, intra-assay CV was <5% for quality controls of 4.2 and 21.7 µg/mL.

Serum insulin concentrations were measured using a commercial ELISA kit validated for feline insulin. Intra and inter-assay reproducibility of feline insulin ELISA kit are less than 10%. Serum triglyceride concentrations were determined using an autoanalyser (Hitachi-7180) using manufacturer’s reagents. The intra-assay and inter-assay CV was <5%. Feline pancreatic lipase concentrations were determined at a commercial laboratory by radioimmunoassay validated for use in the cat. Concentrations of MCP-1 were determined using a canine MCP-1 ELISA kit. Cross-reactivity with feline plasma samples was shown, and serial dilution (factors were x1, x2 and x4 ) of two samples at 163.8 and 316.9 pg/ml resulted in correlation coefficients of 0.998 and 1.000 respectively. The intra-assay CV was <5% for both samples.

**Statistical analyses:**

The effect of breed (non-Burmese versus Burmese) on measures associated with abnormal glucose metabolism, diabetes, and obesity (screening, fasting and 2 h blood glucose
following a glucose tolerance test, leptin, adiponectin, leptin: adiponectin ratio, fPLi, insulin, glucose: insulin ratio, triglyceride, and MCP-1) was analysed by unpaired t-test. Data were log transformed to remove heterogeneity of variance, as required.

Using the metabolomics package of R\textsuperscript{n}, metabolite data were normalized using a single internal standard method where a normalized data matrix is obtained by subtracting the log metabolite abundance of a single internal standard from the log abundances of the metabolites in each sample\textsuperscript{41}. A linear regression model (adjusting for the covariances of body condition, sex and age) was used to identify and rank significant GC-MS metabolites. Pairwise comparisons were made (unpaired t test) and P-values are presented as both unadjusted and adjusted using the Benjamini-Hochberg step-up false discovery rate method\textsuperscript{42}. P-values <0.05 were considered significant.

Pearson correlations were used to examine the relationships between significant identified GC-MS metabolites and measures associated with abnormal glucose metabolism and diabetes. These potential markers of diabetic predisposition included blood glucose (screening, fasting, and 2 h blood glucose following a glucose tolerance test), insulin, triglyceride, adiponectin, leptin, fPLi, and MCP-1 concentrations. Indices such as glucose:insulin, and leptin:adiponectin ratios were also calculated.
Results

Metabolomic analytes

GC-MS analyses identified 173 metabolites, 64 of which were found to be significantly different (P<0.05) between Burmese and non-Burmese cats, when adjusted for body condition score, age and sex. RLA plot medians were close to zero and had acceptable variation (Figure 8.1). Of these 64 metabolites, 23 were identified using the laboratory’s metabolomic library and comprised of 10 amino acids, 3 fatty acids and sterols, 6 acids and 2 sugars. (Figure 8.2, Table 8.1). Identified non-significant metabolites are listed in Appendix 8.1.

Amino acids constituted the largest proportion of metabolites that were different (P<0.01) between Burmese and non-Burmese cats (Table 8.1). Some glucogenic amino acids were increased in Burmese cats: alanine, cysteine, arginine, proline, and glutamine. The aromatic, glucogenic and ketogenic amino acids phenylalanine and tyrosine were both increased (P<0.01). Hydroxyproline and aminobutyric acid were also increased (P<0.01), whilst beta-alanine was decreased (P<0.00001) in Burmese cats. The branched chain amino acids (valine, leucine, isoleucine) and threonine were found to be not significantly different between non-Burmese and Burmese cats (data not shown). Urea, the breakdown product of protein metabolism, was increased (P<0.05) in Burmese cats.

In Burmese cats, the fatty acids monooctadecanoylglycerol and octadecanoic (stearic) acid, and cholesterol were decreased (P<0.05) while palmitoleic, palmitic, and tetradecenoic acids were not significantly different. The sugars fructose and glucose, were both decreased (P<0.01) in Burmese cats. Many acids were significantly different between the two groups. Glycerate, glycolic acid, lactic acid, phosphoric acid, succinate, and threonic acid were all
decreased (at least p<0.05) in Burmese cats. Also, glycerol-3-phosphate, the phosphoric ester of glycerol, was decreased (p<0.00001) in Burmese cats (Table 8.1).

**Insulin, triglyceride, adipokines, MCP-1 and fPLi**

We have previously reported there were no significant differences between Burmese and non-Burmese cats in measures associated with glucose metabolism determined using a glucose meter, including blood glucose (screening, fasting and 2-h blood glucose in GTT) (Thesis Chapter 5 and 6) (Figure 8.2). However, in Burmese cats, insulin concentrations were higher, and the insulin to glucose ratio lower (P<0.05), which was accentuated by a trend to lower fasting glucose concentrations (P=0.09) (Figure 8.2). For the adipokines leptin and adiponectin, there were no differences between Burmese and non-Burmese cats, although MCP-1 was higher (P<0.05). However, cholesterol (by metabolomic analysis) (P<0.05), triglyceride (by biochemical assay) (P<0.01), and fPLi (by commercial RIA kit) approached being significantly higher (P=0.06) in Burmese cats (Figure 8.2). Triglyceride was higher (P<0.01) and total cholesterol was lower (P<0.05) in Burmese, and there was no significant sex effect on either (P=0.33 and P=0.72, respectively). There also was no interaction of breed by sex (P=0.37) for both total cholesterol and triglyceride.

**Associations between GC-MS metabolites and measures of abnormal glucose metabolism and diabetes**

Numerous relationships were identified between GC-MS metabolites and measures of glucose metabolism when Burmese and non-Burmese cats were pooled (Table 8.2). The amino acids cysteine, glutamine, hydroxyproline, phenylalanine and tyrosine, were all positively correlated with insulin, whilst B-alanine was negatively correlated with insulin (P=0.05). Arginine, phenylalanine, and tyrosine were all positively correlated with
triglyceride. Phenylalanine was negatively and lactic acid positively correlated with glucose:insulin ratio. Urea was positively and monooctadecanoylglycerol negatively associated with fPLi. Alanine and proline had a positive, whilst glycerol-3-phosphate and glycolic acid had a negative association with MCP-1 (Table 8.2). Glucose identified in metabolomics was positively correlated with fasting blood glucose measured by glucose meter.
Discussion

Burmese cats are reported to be 4 times more likely to develop diabetes than domestic cats\(^2,5\). However, standard measures of glucose homeostasis such as fasting glucose and glucose tolerance were not different between Burmese and non-Burmese cats. In this study, we compared the metabolomic profiles of healthy senior Burmese and non-Burmese cats, and important findings were that 64 metabolites were present in different qualities in Burmese cats, of which 23 were known metabolites comprising of 10 amino acids, 3 fatty acids and sterols, 6 acids and 2 sugars and 2 other compounds. Monocyte chemoattractant protein was also increased and consistent with the literature, triglyceride concentrations in Burmese were also increased.

**Alterations in fatty acid metabolism**

Consistent with other reports in the literature, Burmese cats in our cohort had increased triglyceride concentrations on standard biochemical analysis. Lean Burmese in Australia and Europe have increased fasting triglyceride concentrations, decreased ability to clear triglycerides after an oral fat challenge or a high fat meal and an exaggerated increase in obesity-induced dysregulation of lipid metabolism\(^2,3\). These abnormalities may be associated with their increased risk of diabetes\(^2,3\). In our cohort, there was no effect of sex or interaction between breed and sex on triglyceride concentrations, although sample size was small. This is in contrast to one study also with small sample size, which reported that only lean male Burmese had higher VLDLs (very low-density lipoproteins), which are the main triglyceride-carrying component of blood, and females did not show this pattern\(^2\).

Total cholesterol represents the sum of the cholesterol carrying components in blood - LDL (low-density lipoprotein), HDL (high-density lipoprotein) cholesterol, and VLDL, with LDL
carrying the largest proportion of cholesterol\textsuperscript{44}. Although total cholesterol was decreased in our cohort of senior Burmese cats, it has been previously reported that total cholesterol is not different between Burmese and non-Burmese\textsuperscript{10,22}, and this contrasts with the increased total cholesterol in humans with IFG and diabetes\textsuperscript{45}. However, lean male Burmese cats are reported to have lower HDL as measured by their cholesterol lipoprotein fraction, albeit sample size was small (n=5)\textsuperscript{22}. The lower total cholesterol concentration in our cohort might reflect lower HDL concentrations, or reflect our small sample size. In our cohort of cats, there was effect of sex or interaction between breed and sex with total cholesterol, agreeing with a study of young cats (age range 1.5-8.6 years)\textsuperscript{10}.

Monooctadecanoylglycerol and stearic (octadecanoic) acid were significantly decreased in Burmese cats. These findings are novel in cats and are contrary to findings in humans with impaired fasting glucose (prediabetes) and diabetes\textsuperscript{45,46}, where stearic acid is significantly increased. In isolated mouse islets, stearic acid increased basal insulin secretion and potentiated glucose-induced insulin secretion\textsuperscript{47}. In Burmese cats, stearic acid and insulin concentrations were not associated. Stearic acid is either obtained from the diet or synthesised by elongation of palmitate. Both palmitate and stearate are the major substrates for the enzyme stearoyl-CoA desaturase-1, with stearate being converted to oleate which is the preferred substrate for triglyceride synthesis. Lower stearate might reflect increased activity of SCD1, and increased formation of triglycerides as we noted in Burmese cats. Indeed, SCD1 is an important target for the adipokine hormone leptin, and thought in part to mediate the anorexigenic effect of leptin\textsuperscript{48}. We did not find an association between leptin and stearic acid (Appendix table 8.2), nor differences in plasma leptin concentrations between Burmese and non-Burmese cats. Research on triglyceride synthesis in Burmese cats, including stearic acid and the enzyme SCD1 warrants further investigation.
Alterations in amino acids and derivatives

Our study identified 10 metabolites associated with amino acid metabolism which were significantly different between Burmese and non-Burmese cats. Eight BCAAs were increased in Burmese cats - alanine and phenylalanine, tyrosine, glutamine, proline, hydroxyproline, arginine, and cysteine. This is consistent with the finding in non-obese humans with increased insulin resistance\(^{45,49}\) where serum BCAAs are increased, and is hypothesized to result from increased protein catabolism together with impaired BCAA metabolism in liver and adipose tissue associated with insulin resistance\(^{45,50}\).

Tyrosine and phenylalanine

Phenylalanine a gluconeogenic and ketogenic aromatic amino acid, and tyrosine the precursor of phenylalanine were both these amino acids were increased in our cohort of Burmese cats. Such increases in phenylalanine and tyrosine concentrations are associated with insulin resistance and type 2 diabetes in humans\(^{49,51-54}\). In humans, increased concentrations of phenylalanine (males and females) and tyrosine (males only) predicted insulin resistance indicated by HOMA at a 6-yr follow-up\(^{55}\). In our cats, we found a positive moderate correlation between these amino acids and insulin concentrations, and moreover increased fasting insulin is considered an indicator of insulin resistance in cats\(^{56}\). A study in humans reported no correlation between fasting glucose and phenylalanine\(^{55}\), similar to the findings in our cohort of cats. The authors concluded that altered amino acid metabolism may be more related to insulin resistance before glucose concentrations were affected. In humans, insulin resistance and future risk for type 2 diabetes is also predicted by a distinct amino acid pattern of increased isoleucine, leucine, and valine\(^{55}\), but these were not significantly different in our
Burmese cohort. Further studies are needed to determine whether increases in the aromatic amino acids tyrosine and phenylalanine also predict future diabetes in cats.

**Alanine and glutamine**

Alanine and glutamine were increased in our cohort of Burmese cats. These gluconeogenic amino acids have been associated with insulin resistance\(^57\). In our cats, there was a strong positive correlation between glutamine (but not alanine) and insulin, and no association of either amino acid with fasting glucose. Humans with IFG and untreated diabetes have significantly increased glutamine\(^45\), and an association between IFG and type 2 diabetes with alanine has also been reported\(^45\). In cats in diabetic remission these amino acids are reduced (Gottleib *et al*., unpublished data).

Increased concentrations of BCAA lead to increases in by-products of BCAA catabolism such as alanine\(^45,51\). Alanine is formed from glutamate by the liver enzyme alanine aminotransferase (ALT). In patients with type 2, changes in BCAA are hypothesized to increase liver enzymes\(^58\), and increased ALT levels are a predictor of diabetes in humans. Measurement of ALT is recommended to be included in screening panels for diabetes\(^59\). Our cats all had normal ALT measured on a routine biochemistry panel.

**Beta-alanine**

Beta-alanine was the only amino acid significantly decreased in Burmese cats, and exhibited a negative correlation with insulin. In humans with type 2 diabetes, beta-alanine supplementation has been shown to reduce fasting blood glucose concentrations, and was hypothesized to increase insulin secretion and/or improve glucose-uptake\(^60\). In our cohort of cats, however there was no correlation between beta-alanine and glucose concentrations.
Arginine

Arginine is a gluconeogenic amino acid and often used in insulin stimulation tests in cats because arginine is a potent stimulus for insulin secretion\textsuperscript{61}. Several studies have reported that arginine has stronger insulinotropic effects than alanine\textsuperscript{62,63}. Arginine was significantly increased in Burmese cats, but there was no correlation with insulin or any measure of glucose metabolism. However, there was a strong correlation between arginine and triglycerides (P<0.01). L-arginine was reported to enhance the effects of statins in 33 hypertriglyceridaemic patients by lowering triglycerides but there was no decrease in triglycerides when given alone\textsuperscript{64}. L-arginine administration was also reported to decrease triglycerides in diabetic rats\textsuperscript{65} but no similar findings have been reported in cats.

Proline and hydroxyproline

Proline and hydroxyproline were significantly increased in our cohort of Burmese cats, and both were also positively correlated with fasting insulin. Hydroxyproline is produced by hydroxylation of proline\textsuperscript{66} and hydroxyproline is increased in human type 2 diabetic patients\textsuperscript{67} and nondiabetic obese subjects\textsuperscript{68}. Hydroxyproline is a major component of collagen\textsuperscript{66}, and collagen is increased in the skeletal muscle of these subjects, suggesting that hydroxyproline and collagen may play a role in insulin resistance in muscle\textsuperscript{68}.

Other acids altered between Burmese and non-Burmese cats

Lactic, threonic and phosphoric acids were all decreased in Burmese cats whilst aminobutyric acid was increased. In humans with increased insulin resistance, lactate production is decreased\textsuperscript{69}, and it was also decreased in our cohort of Burmese cats. Although there was no significant relationship between lactic acid and insulin in our cohort of Burmese cat, there
was a positive correlation with the glucose:insulin ratio, consistent with the finding in humans\textsuperscript{69}.

\textit{Alterations in carbohydrate metabolism}

\textit{Fructose and glucose}

Burmese cats had decreased fructose compared to non-Burmese cats. In humans, increased serum fructose is hypothesized to have a causative role in insulin resistance\textsuperscript{45,70} and hyperlipidemia\textsuperscript{71}. In our study, fructose had no correlation with insulin or the glucose:insulin ratio. As reported previously (Chapter 5), glucose concentrations in all our Burmese cats were within the normal reference range. In the smaller sample group used for this current study, glucose was significantly decreased on metabolomic analysis and trended towards significantly decreased when measured with a portable glucose monitor. Glucose identified in metabolomics was positively correlated with fasting blood glucose measured by glucose meter.

\textit{Succinate and glycerate}

Succinate and glycerate, an ester of glyceric acid was decreased in Burmese cats but glyceric acid, involved in the conversion of glucose to pyruvate, was not significantly different between Burmese and non-Burmese cats. Succinyl-coA is generated from succinate and the subsequent metabolite mevalonate, triggers insulin release in isolated rat pancreatic islets\textsuperscript{72}. However, neither succinate no glycerate correlated with insulin in our cohort of cats.

\textit{Glycerol-3-phosphate}

Glycerol-3-phosphate is a phosphoric ester of glycerol, and glycerol-3-phosphate is increased by stimulation of glucose transport by insulin\textsuperscript{73}. Insulin also inhibits breakdown triglyceride
into glycerol and fatty acids\textsuperscript{74}. Glycerol-3-phosphate was decreased in Burmese cats and there was no correlation between glycerol-3-phosphate and insulin.

**Alterations in urea**

Urea was significantly higher in Burmese cats than non-Burmese cats. All the study cats had blood urea levels within laboratory reference range, and none were diagnosed with renal disease based on serum urea and creatinine concentrations within the laboratory reference range. Similar results were found in cats in diabetic remission (Gottlieb, unpublished data). The increase in urea may be due to an increase in alanine metabolism through the glucose-alanine cycle, where pyruvate is converted to alanine in the muscle and subsequently back to pyruvate in the liver by the TCA cycle because urea is a byproduct\textsuperscript{75}. Alanine levels were also significantly increased in Burmese cats, and therefore, the increased urea levels may be due to increased alanine production in the muscle. In human males, urea, along with 10 other discriminators, were found to be biomarkers of impaired fasting glucose\textsuperscript{76}, but a similar relationship was not found in our cat study. Further pyruvate was not increased in Burmese cats.

**MCP-1**

A novel finding in our study was that MCP-1 was significantly increased in Burmese cats. MCP-1 suppresses production of adiponectin\textsuperscript{77} and increased MCP-1 concentrations occur with obesity and are associated with type 2 diabetes in humans\textsuperscript{78-83}. In humans and mice, MCP-1 induces inflammation in adipose tissue\textsuperscript{77,84-86}. In severely obese human patients, MCP-1 protein expression was higher in omental fat than in subcutaneous fat, and was associated with increased macrophage infiltration into omental fat\textsuperscript{87}. However, MCP-1 concentrations were not increased in obese non-Burmese cats (Thesis Chapter 7). As
Burmese cats become obese, they develop a characteristic spherical appearance associated with prominent deposition of abdominal fat, rather than prominent subcutaneous inguinal fat deposits that domestic cats develop (per observations Rand), possibly making MCP-1 expression more likely in Burmese cats. Increased MCP-1 concentrations are associated with decreased insulin sensitivity\textsuperscript{80,86,88}, and our cohort of Burmese cats had increased insulin concentrations, and decreased glucose: insulin ratio, indicative of insulin resistance. Because of the effect of MCP-1 on insulin sensitivity, further investigation is warranted to determine if MCP-1 plays a role in the predisposition of Burmese to diabetes.

**Limitations of the study**

A limitation of our study was that adequate dietary information was only available for 6/19 (32\%) of the Burmese and 16/30 (53\%) of the non-Burmese cats in our study. Dietary macronutrients have been shown to influence some of the biomarkers assessed in our study. For example, in cats, some amino acids and markers of lipid metabolism increase with diets of different macronutrient composition, specifically high protein (50\% ME) and high fat (50\% ME) diets respectively, but markers of carbohydrate and energy metabolism are less affected\textsuperscript{63}. In our study, mean dietary protein and carbohydrate content were similar between groups, but dietary fat was higher in the Burmese cats. It is recommended that for future studies, cats should be on the same diet, or at least detailed dietary information should be available for inclusion in the statistical analysis. This was, however, difficult due to all cats being client-owned and collected information, though available for all cats, was unreliable. Only 23 of 64 metabolites that were different between Burmese and non-Burmese were identified. Additionally, the changes observed, although significant, are small and because the study used an untargeted approach and thus it is difficult to predict what the observed increased or decreased relative changes may mean physiologically. A future direction to this
research would be a targeted metabolomic approach allowing quantitative measurements and identification of unknown metabolites that were significantly different in Burmese from non-Burmese cats. Additionally, a larger cohort of cats with a more similar proportion of obese cats in each group would be useful to validate our findings. Our study was a cross-sectional study, and a longitudinal study is necessary to determine if some of the metabolites which were different in Burmese cats are indeed predictors of diabetes in cats.
Conclusion

This study is the first to report metabolite differences between healthy non-Burmese and Burmese cats and it identified some biomarkers involved in amino acid, carbohydrate and lipid metabolism that are altered in Burmese cats, some of which are recognized to increase the risk of diabetes in humans. Further research is required to determine if these are useful as markers of metabolic dysfunction in cats at risk of developing diabetes.

Funding

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Acknowledgements

The authors would like to thank the Cat Clinics, Greencross Veterinary Clinics, Small Animal Hospital UQ St Lucia, participating owners and cats and Metabolomics Australia

Footnotes

a Idexx Laboratories, Brisbane Australia.
b Abbott Alpha Trak©
c Linco's Multi-Species Leptin Radioimmunoassay (RIA) Kit, Linco Research Inc, St Charles, MO
d B-Bridge international, Otsuka, Tokyo, Japan
e Nippon University
240

f Morinaga Institute of Biological Science, Kanagawa, Japan

g $^{13}$C$_6$-sorbitol/$^{13}$C$_{18}$N-Valine in water, 0.2 mg mL$^{-1}$

h 0.029% (v/v) n dodecane, n-pentadecane, n-nonadecane, n-docosane, n-octacosane, n-dotriacontane, n-hexatriacontane dissolved in pyridine

i Gerstel 2.5.2 autosampler, a 7890A Agilent gas chromatograph and a 5975C Agilent quadrupole MS (Agilent, Santa Clara, USA).

j Varian, Inc, Victoria, Australia

k Agilent MassHunter Workstation Software, Quantitative Analysis, Version B.05.00/Build 5.0.291.0 for GCMS

l Max-Planck-Institute for Plant Physiology, Golm, Germany (http://csbdb.mpimp-golm.mpg.de/csbdb/dbma/msri.html)

m ($^{13}$C$_6$H$_4$O$_6$ – Sorbitol - (0.5 mg/mL), $^{13}$C$_5$-$^{15}$N-Valine - (0.5 mg/mL), 2-aminoanthracene - (0.25 mg/mL) Pentafluorobenzoic acid - (0.25 mg/mL))


o GraphPad Prism version 7.01. GraphPad Software, Inc. La Jolla, CA, USA.

Reference Interval Draft Version, Copyright 2005, University of Cincinnati
### Table 8.1. Significant metabolites from GC-MS analysis of Burmese and non-Burmese senior cats. Values are expressed as log<sub>e</sub> fold-change of Burmese relative to the Non-Burmese (control) group. P-values from unpaired t-test comparisons are presented as unadjusted, and adjusted using the Benjamini-Hochberg step-up false discovery rate method. P-values <0.05 were considered significant.

<table>
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<tr>
<th>Metabolite</th>
<th>Log&lt;sub&gt;e&lt;/sub&gt; fold change</th>
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<th>Adjusted P-value</th>
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<td>&lt;0.01</td>
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<tr>
<td>Glutamine</td>
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<tr>
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<td>Proline</td>
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<tr>
<td>Phosphoric acid</td>
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<td>Succinate</td>
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<td>-0.250</td>
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<td>Monooctadecanoylglycerol</td>
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<th>Other compounds</th>
<th>Glycerol-3 phosphate</th>
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<th></th>
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<td>0.168</td>
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<td>Urea</td>
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<td>0.066</td>
<td>&lt;0.01</td>
<td>0.02</td>
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</table>
Table 8.2. Pearson correlations of significant identified metabolites from GC-MS analysis and measures of abnormal glucose metabolism and diabetes for all cats (Burmese and non-Burmese). P-values <0.05 were considered significant

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Measure of abnormal glucose metabolism and diabetes</th>
<th>R-value</th>
<th>P-values</th>
</tr>
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<tbody>
<tr>
<td><strong>Amino Acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>MCP-1</td>
<td>+0.30</td>
<td>0.04</td>
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<tr>
<td>Arginine</td>
<td>Triglyceride</td>
<td>+0.41</td>
<td>&lt;0.01</td>
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<tr>
<td>B-alanine</td>
<td>Insulin</td>
<td>-0.28</td>
<td>0.05</td>
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<td>Cysteine</td>
<td>Insulin</td>
<td>+0.38</td>
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<td>Insulin</td>
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<td>Insulin</td>
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<td>Insulin</td>
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<td>&lt;0.01</td>
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<td>Triglyceride</td>
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<td>0.02</td>
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<td>Insulin</td>
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<td>Tyrosine</td>
<td>Insulin</td>
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<td><strong>Acids</strong></td>
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<td>Compound</td>
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<tr>
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<td>---------</td>
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<tr>
<td>Glycolic acid</td>
<td>MCP-1</td>
<td>-0.33</td>
<td>0.02</td>
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<tr>
<td>Lactic acid</td>
<td>Glucose:insulin ratio</td>
<td>+0.41</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Fatty acids and Sterols</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mono-octadecanoylglycerol</td>
<td>fPLi</td>
<td>-0.29</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Sugars</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>Fasting glucose</td>
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<td>0.04</td>
</tr>
<tr>
<td><strong>Other compounds</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol-3-phosphate</td>
<td>MCP-1</td>
<td>-0.31</td>
<td>0.03</td>
</tr>
<tr>
<td>Urea</td>
<td>fPLi</td>
<td>+0.41</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
Figures

Figure 8.1: Relative log abundance (RLA) plot where the median of each metabolite in the data matrix is visualized, and then subtracted this median from each metabolite. Red bars are Burmese and blue bars non-Burmese cats.
Figure 8.2. Bar Charts showing means, standard error of the means, and p-values calculated by unpaired t-test of measures associated with glucose metabolism and diabetes in non-Burmese (n=30) and Burmese (n=19) senior cats. ns is non-significant (p>0.10), * is significant at P<0.05, ** is significant at P<0.01.
References


57. Stancakova A, Civelek M, Saleem N. Hyperglycemia and a common variant of GCKR are associated with the levels of eight amino acids. *Diabetes* 2012;61:1895-1902.


83. Zietz B, Büchler C, Herfarth H, et al. Caucasian patients with type 2 diabetes mellitus have elevated levels of monocyte chemoattractant protein-1 that are not influenced by the −2518 A-->G promoter polymorphism. *Diabetes, Obesity and Metabolism* 2005;7:570-578.


Chapter 9: Conclusion

The findings of this thesis have established and validated tests for screening, impaired fasting glucose and glucose tolerance in senior cats; the tests are easy to perform and designed to be used in a clinical setting. This thesis has also contributed to our understanding of the metabolic differences in cats at high risk of developing diabetes- namely obese and Burmese senior cats. The research presented in this thesis paves the way for early clinical diagnosis of prediabetes in cats, which may help prevent clinical disease in the future.

Volume of distribution does not increase linearly with bodyweight and could lead to overweight and obese cats being incorrectly classified as having impaired glucose tolerance when interpreting the results of a glucose tolerance test. In chapter 4 (“Dosing obese cats based on body weight spuriously affects some measures of glucose tolerance”. Domest Anim Endocrinol. 2016 Oct;57:133-42), a retrospective study was performed using glucose concentration data from glucose tolerance and insulin sensitivity tests before and after cats were fed ad libitum for 9 to 12 months to promote weight gain. An important finding was that dosing glucose by bodyweight results in spurious effects on some measures of glucose tolerance in obese cats. Equations were developed to adjust either glucose dose or 2-h glucose to compensate for the volume of distribution effects of obesity. Based on these results, a glucose tolerance test requiring only 2 blood samples was standardised and validated for cats of all body condition scores, ranging from lean to obese. This feline equivalent to the human GTT provides clinical researchers and veterinary practitioners with a tool for identifying glucose intolerance in cats. This study demonstrated that testing of overweight and obese cats requires an adjustment to compensate for the ‘dosage’ effects of obesity, following guidelines outlined in this research. These findings have important implications for clinical studies assessing the effect of interventions on glucose tolerance when lean and obese cats are compared.
Evidence-based cutpoint values are important for diagnosing prediabetes in at risk cats, such as obese and Burmese cats. Because cats with impaired fasting glucose or glucose intolerance are at increased risk of diabetes, prediabetic cats need to be identified so management regimes can be implemented, including weight loss and dietary intervention. In chapter 5 (“Diagnosis of prediabetes in cats: glucose concentration cut points for impaired fasting glucose and impaired glucose tolerance” Domest Anim Endocrinol. 2016 Oct;57:55-62. doi: 10.1016/j.domaniend.2016.05.008. Epub 2016 May 26.), the methodology and cutpoints were established for fasting and 2-h blood glucose concentrations following the simplified intravenous glucose tolerance test designed in Chapter 4 for use in healthy cats 8 years and older with a range of body condition scores using ear/ paw samples and a portable glucose meter calibrated for feline blood. The upper cutpoint for fasting glucose was 117 mg/dL (6.4 mmol/L) and for 2-h glucose was 176 mg/dL (9.8 mmol/L). Burmese had significantly higher 2-h blood glucose concentrations at the higher dose rate, suggesting relative intolerance to glucose at higher doses and this warrants further investigation.

Casual blood glucose, defined as blood glucose measured unrelated to time of eating or type of food, is used as a screening test to diagnose diabetes in humans. Screening blood glucose measurement in cats represents a simple test, designed for use in a clinical setting. In Chapter 6 (Cutpoints for screening and fasting blood glucose concentrations in healthy senior cats, Journal of Feline Medicine and Surgery, 2017, doi.org/10.1177/1098612X16685675), the measurement of screening blood glucose using a portable glucose meter validated for feline blood was standardized and the upper cutpoint of 188 mg/dL (10.5 mmol/L) established for ideal body condition cats. Another important finding was that behaviour score, BCS, fasting blood glucose concentration, and amount of carbohydrate consumed in the 2 to 6 hours before sampling collectively explained very little variability in screening blood glucose. It is recommended to retest cats with values from 117 mg/dL to 188 mg/dL (6.5 mmol/L to 10.5
mmol/L) 3-4 hours later. Cats with initial screening blood glucose > 188 mg/dL (10.5 mmol/L), or a second screening blood glucose ≥117 mg/dL (6.5mmol/L) 3-4 hours after the first, should have fasting blood glucose and our simplified glucose tolerance test performed after overnight hospitalization using methodology developed in this thesis.

Type 2 diabetes is the most common form of diabetes in humans and cats, and obesity is a risk factor. Metabolomic markers of obesity and predictors of diabetes have been identified in humans, but not in cats. In Chapter 7 (Metabolomic differences between lean and obese neutered senior cats and associations with glucose tolerance), metabolomic differences between lean and obese senior cats were identified, and associations of these metabolites with measures associated with glucose metabolism (screening, fasting and 2-h blood glucose following a glucose tolerance test) and diabetes related parameters (insulin, glucose:insulin ratio, leptin triglycerides, adiponectin, leptin:adiponectin ratio, fPLi and MCP-1) were established. Phenylalanine, and numerous fatty acids, glycolic acid and glycerol were increased and amino acid alanine was decreased in obese senior cats. Numerous associations between metabolites and other measures were identified: fatty acids were positively (and alanine negatively) correlated (P<0.05) with leptin, and glycolic acid and glycerol were positively correlated with insulin (P<0.05). The relationship between BCAA and fatty acids and diabetes has been documented in humans and fatty acids have been shown to be independant predictors of progression to diabetes in humans. Our study indicated a similar relationship between phenylalanine and obese cats and its relationship with insulin sensitivity. This study was the first to report the metabolomic markers of feline obesity and the association between metabolomic markers and hormonal variables in healthy senior cats and opened the path to forming a metabolomic footprint for these cats. This could prove useful in diagnosing prediabetes in cats and other species in the future.
As with obese cats, Burmese cats have been shown to be at high risk of developing diabetes. In fact, Burmese cats are 4 times more likely to develop diabetes than domestic cats. Our previous studies showed that Burmese cats may have relative intolerance to glucose at higher doses and others have found inherited alterations in lipid metabolism which may explain this increased risk of developing diabetes. In chapter 8 (Metabolite differences between healthy senior Burmese and non-Burmese cats and associations between metabolites and measures of glucose metabolism) we identified metabolomic differences between Burmese and non-Burmese cats and correlations between metabolites and measures associated with glucose metabolism. BCAA are increased in diabetic humans and associated with insulin resistance, and were also increased in Burmese cats and positively correlated with insulin, suggesting they may contribute to the metabolomic footprint of this breed. This study is the first to report metabolite differences between healthy non-Burmese and Burmese cats. These might prove useful markers of metabolic dysfunction in cats at high risk of developing diabetes, and improve understanding of the metabolic derangements associated with diabetes in Burmese cats. We also found there were some similarities in the expression profile between lean Burmese and obese domestic cats, and that these similarities may facilitate identification of biomarkers of obesity and diabetes risk that could be examined in future studies.

In summary, this thesis has standardized and validated tests for screening blood glucose, impaired fasting glucose and impaired glucose tolerance in senior cats of all body condition scores. These tests are easy to perform and use in a clinical setting. The findings of this thesis also increased our understanding of the effects of obesity on blood glucose and demonstrated how results can be adjusted to allow the same cutpoints to be used in all cats, lean and obese. Additionally, the research in this thesis has identified metabolomic differences in cats at high risk of developing diabetes, namely obese and Burmese cats, which may aid in identifying cats more likely to develop diabetes in the future.
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Appendix Table 5.2 Percentage differences of Medians and Interquartile Ranges comparing the subgroups of client-owned cats aged 8 years and older following an 18 h fast and 2 h after a 0.5g/kg intravenous glucose bolus during a simplified intravenous glucose tolerance test.


Appendix Table 5.4 Adjustment of 2-h blood glucose concentration using equation from retrospective study (Reeve-Johnson, M., et al. (2016). Dosing obese cats based on body weight spuriously affects some measures of glucose tolerance. *Domestic Animal Endocrinology* 57:133-142.) with data from this study of cats with 2-h blood glucose concentrations above reference interval upper limits.

Appendix Document 7.1 Instrumentation, instrument settings and chromatographic conditions for GC-MS analyses.

Appendix Figure 7.2 Volcano Plot of GC-MS data showing metabolites significantly different between obese cats compared to lean cats. Metabolites with log fold changes below
zero are decreased in obese cats, and those above zero are increased. Metabolites shown in grey were not significantly different in obese cats…………………………………………268

Appendix 7.3: List of metabolites identified by the using a public domain mass spectra librarya and the in-house Metabolomics Australia mass spectral library which were not significantly different between lean and obese senior cats……………………………………269

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Appendix Figure 8.1 Volcano Plot of GC-MS results showing metabolites that were different between Burmese and non-Burmese cats, Values on X axis are expressed as loge fold-change of Burmese relative to the Non-Burmese cats. Labels indicate known metabolites. The red line indicates threshold for significance (P<0.05) on Y-axis ………..272

Appendix 8.2: List of metabolites identified by the using a public domain mass spectra librarya and the in-house Metabolomics Australia mass spectral library which were not significantly different between Burmese and non-Burmese senior cats……………………………272

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Appendices

**Appendix Table 5.1:** Means (mmol/L), Standard Deviations (SD), Medians and Interquartile Ranges of blood glucose concentrations following an 18 hour fast and 2 minutes (2-min) and 2 hours (2-hr) following an intravenous glucose bolus of 0.5 g/kg bodyweight during a simplified intravenous glucose tolerance test for subgroups of client owned cats aged 8 years and older.

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<th>Mean</th>
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<th>IQR</th>
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<tr>
<td>Fasting</td>
<td>4.5</td>
<td>0.8</td>
<td>4.6</td>
<td>0.7</td>
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<tr>
<td>2-min</td>
<td>21.4</td>
<td>5.3</td>
<td>23.3</td>
<td></td>
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<tr>
<td>2-hr</td>
<td>6.3</td>
<td>2.1</td>
<td>6.2</td>
<td>2.5</td>
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<td><strong>Non-Burmese (n=61)</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
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<td>Fasting</td>
<td>5</td>
<td>1.6</td>
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</tr>
<tr>
<td>2-min</td>
<td>25</td>
<td>6</td>
<td>25.1</td>
<td></td>
</tr>
<tr>
<td>2-hr</td>
<td>6.6</td>
<td>2.5</td>
<td>5.7</td>
<td>2.9</td>
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<tr>
<td><strong>Lean (n=22)</strong></td>
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</tr>
<tr>
<td>Fasting</td>
<td>5.3</td>
<td>1.7</td>
<td>4.9</td>
<td>0.8</td>
</tr>
<tr>
<td>2-min</td>
<td>23.5</td>
<td>6.4</td>
<td>23.3</td>
<td></td>
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<tr>
<td>2-hr</td>
<td>5.6</td>
<td>1.4</td>
<td>5.3</td>
<td>1.35</td>
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<tr>
<td><strong>BCS 6 (n=8)</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Fasting</td>
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Appendix Table 5.2: Percentage differences of Medians and Interquartile Ranges comparing the subgroups of client-owned cats aged 8 years and older following n 18 h fast and 2 h after a 0.5g/kg intravenous glucose bolus during a simplified intravenous glucose tolerance test.

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<th>% Difference</th>
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<th>IQR</th>
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<td>57</td>
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<tr>
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<td>32</td>
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</tr>
<tr>
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<tr>
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<td>3</td>
<td>12</td>
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</tr>
<tr>
<td>2-h</td>
<td>32</td>
<td>27</td>
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<td>Burmese vs Obese</td>
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<td>Non-Burmese Obese vs BCS 7</td>
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<th>Adjustments for 2-h concentrations</th>
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<th>Method a</th>
<th>Method b</th>
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<td>11.3</td>
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<td>10.9</td>
<td>10.1</td>
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Appendix Table 5.4: Adjustment of 2-h blood glucose concentration using equation from retrospective study (Reeve-Johnson, M., et al. (2016). Dosing obese cats based on body weight spuriously affects some measures of glucose tolerance. *Domestic Animal Endocrinology* 57:133-142.) with data from this study of cats with 2-h blood glucose concentrations above reference interval upper limits.

<table>
<thead>
<tr>
<th>Adjustments for 2-h concentrations</th>
<th>Actual 2-h concentration</th>
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<th>Method a</th>
<th>Method b</th>
</tr>
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<tr>
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<td>6</td>
<td>11.3</td>
<td>9</td>
<td>11.1</td>
<td></td>
</tr>
</tbody>
</table>
Appendix 7.1 Instrumentation, instrument settings and chromatographic conditions of GC-MS analyses

**Instrumentation:** Agilent 6520A ESI-QqTOF-MS with attached Agilent 1200 series HPLC System comprised of Degasser, Pump, Auto-sampler with attached chiller, Diode Array Detector.

**Instrument settings:** Gas Temperature = 300°C; Drying Gas = 10 L/min; Nebulizer = 40-45 psig; VCap = 3500V; Fragmentor = 150V, Skinner = 65V, OCT 1 RF Vpp = 750V. Acquisition range 70-1700 m/z, Rate = 1.5-3 spectra/s. Agilent Reference Mass solution co-infused through dual ionisation source for online mass calibration. Each sample was run on positive and negative mode. Autosampler cooled to 7°C.

**Chromatographic Conditions:**

**Reverse Phase:** Zorbax Eclipse XDB-C18, 2.1 mm x 100 mm, 1.8 µm (Agilent) column; solvent (A) is 0.1% formic acid in Milli-Q water and solvent (B) is 0.1% formic acid in CH3CN; a solvent Flow rate: 0.4 mL/min with column temperature of 40°C; Gradient: A 10 min linear gradient from 5% solvent (B) to 100% solvent (B), followed by a 2 min hold at 100% solvent (B), then 5 min re-equilibration at 5% solvent (B) (total time of 17 minutes). Injection volume: 5 µL.

**ANP/HILIC:** Cogent diamond Hydride 2.1 mm x 100 mm, 4 µm particle size (MicroSolv Technology, Brisbane, Australia) column; solvent (B) 90% ACN v/v with 0.1% ammonium acetate w/v and 0.1% acetic acid v/v; solvent (A) 100% deionised water with 0.1% ammonium acetate w/v and 0.1% acetic acid v/v (pH 3.4); solvent flow rate of 0.4 mL/min with column temperature at 50°C; Gradient: starting at 100% solvent (B) then linearly decreasing to 40% solvent (B) over 10 min, followed by a 1 min hold at 40% solvent (B) then re-equilibration at 100% solvent (B) for 6 min (total time is 17 min). Injection volume: 5 µL.
Abbreviations:

ESI-QqTOF-MS = Electrospray ionisation quadrupole time of flight mass spectrometer

HPLC = High Performance Liquid Chromatography

**Appendix Figure 7.2:** Volcano Plot of GC-MS data showing metabolites significantly different between obese cats compared to lean cats. Metabolites with log₂ fold changes below zero are decreased in obese cats, and those above zero are increased. Metabolites shown in grey were not significantly different in obese cats.
Appendix 7.3: List of metabolites identified by the using a public domain mass spectra library and the in-house Metabolomics Australia mass spectral library which were not significantly different between lean and obese senior cats

2-amino Malonic acid
1-Monooc-tadecanoylglycerol
2-amino Butyric acid
2-amino- Malonic acid
2-keto-L-Gluconic acid
4-hydroxy Proline
9,12-Octadecadienoic acid
9-Octadecenoic acid
Arginine
Asparagine
beta-Alanine
beta-sitosterol
Campesterol
Cholesterol
citric acid
Cysteine
Erythronic acid
Fructose
Glucose
Glutamate
Glutamine
Glycerate
Glycerol-3-Phosphate
Glycine
Heptadecanoic acid
Hexadecanoic acid
Hexadecenoic acid
Isoleucine
Lactic acid
Leucine
Lysine
Malic acid
Monomethylphosphate
myo Inositol
Octadecanoic acid
Phosphoric acid
Phosphoric acid monomethylester
Proline
Pyroglutamic acid
Pyruvic acid
scylo Inositol
Serine-3TMS
Succinate
Threitol
Threonic acid
Threonine
Tyrosine
Urea
Valine
Appendix Table 7.4 Pearson correlation of significant metabolites from GC-MS analysis of Lean and Obese senior cats and measures associated with abnormal glucose metabolism and diabetes. Correlations are for both lean and obese cats (n=35). P- values <0.05 were considered significant.

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<tr>
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**Appendix Figure 8.1** Volcano Plot of GC-MS results showing metabolites that were different between Burmese and non-Burmese cats. Values on X axis are expressed as log$_e$ fold-change of Burmese relative to the Non-Burmese cats. Labels indicate known metabolites. The red line indicates threshold for significance (P<0.05).

**Appendix 8.2:** List of metabolites identified by the using a public domain mass spectra library$^a$ and the *in-house* Metabolomics Australia mass spectral library which were not significantly different between Burmese and non-Burmese senior cats

- 2-amino Malonic acid
- 2-amino- Malonic acid
- 2-keto-L-Gluconic acid
9,12-Octadecadienoic acid
9-Octadecenoic acid
9Z-Hexadecenoic acid
Asparagine
beta-sitosterol
Campesterol
citric acid
Erythronic acid
Glutamate
Glycerol
Glycine
Heptadecanoic acid
Hexadecanoic acid
Hexadecenoic acid
Isoleucine
Leucine
Lysine
Malic acid
Monomethylphosphate
myo Inositol
Phosphoric acid monomethylester
Pyroglutamic acid
Pyruvic acid
scyllo Inositol
Serine-3TMS
Tetradecanoic acid
Threitol
Threonine
Valine
## Appendix Table 8.3

Pearson correlation of significant metabolites from GC-MS analysis of Burmese and non-Burmese senior cats and known measures of glucose metabolism for all cats. P-values <0.05 were considered significant (P=ns not significant, p>0.1)

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<th>Metabolite</th>
<th>Screening Blood Glucose</th>
<th>Fasting Blood Glucose</th>
<th>2-h Blood Glucose</th>
<th>Leptin</th>
<th>Adiponectin</th>
<th>L:A ratio</th>
<th>fPLi</th>
<th>Insulin</th>
<th>Glucose:Insulin ratio</th>
<th>Triglyceride</th>
<th>MCP-1</th>
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<td>r=+0.16</td>
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<td>p=ns</td>
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<td>r=+0.05</td>
<td>r=+0.15</td>
<td>r=-0.24</td>
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