Evaluation of Nesfatin-1 Expression in Lean, Overweight, and Diabetic Cats

A Thesis Submitted to the
College of Graduate and Postdoctoral Studies
In Partial Fulfillment of the Requirements
For the Degree of Master of Science
In the Department of Veterinary Pathology
University of Saskatchewan
Saskatoon

By

Peter Toh

Permission to Use

In presenting this thesis in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Disclaimer

References in this thesis/dissertation to any specific commercial products, process, or service by trade name, trademark, manufacturer, or otherwise, does not constitute or imply its endorsement, recommendation, or favoring by the University of Saskatchewan. The views and opinions of the author expressed herein do not state or reflect those of the University of Saskatchewan and shall not be used for advertising or product endorsement purposes.

Requests for permission to copy or to make other uses of materials in this thesis/dissertation in whole or part should be addressed to:

Head of the Department of Veterinary Pathology
52 Campus Drive
University of Saskatchewan
Saskatoon, Saskatchewan S7N 5B4 Canada

OR

Dean
College of Graduate and Postdoctoral Studies
University of Saskatchewan
116 Thorvaldson Building, 110 Science Place
Saskatoon, Saskatchewan S7N 5C9 Canada

Acknowledgements

Many individuals made major contributions to the creation of this thesis, and I would not have been able to complete this manuscript without all their assistance. I would first like to thank the members of my advisory committee: Elisabeth Snead, Nicole Fernandez, Ryan Dickinson, Andy Allen, Elemir Simko, and Melissa Meachem. I would like to particularly thank Dr. Snead for helping to recruit our study samples and being available at all times to assist with sample collection's, and my supervisor Dr. Meachem for all her support and guidance in the formulation and execution of the research, and preparation of this manuscript. I would also like to thank Dr. Chantal McMillan at the University of Calgary for her generous donation of several tissue and plasma samples.

At the Western College of Veterinary Medicine, I would like to thank the staff and students in the college and Veterinary Medical Centre for allowing their pets to be a part of our study. Special thanks to the nurses and technicians in the Veterinary Medical Centre, especially Laura Schroeder and Brenda Beierle for their help in the collection of serum and plasma samples. In the veterinary pathology department, I thank all the laboratory technicians who provided invaluable technical assistance. In particular, I would like to thank Betty Chow-Lockerbie for her assistance in running and trouble-shooting all aspects of the PCR's performed, and LaRhonda Sobchisin for her assistance in running and trouble-shooting the ELISA's. I also thank Ben Elwood and Afzal Javed for their assistance.

Outside of the veterinary college, several veterinarians assisted with the recruitment and referral of diabetic cats for this manuscript, often at short notice. I thank Dr. Sue Tedesco and her staff at Forest Grove Veterinary Clinic, as well as Drs. Ira Froimovitch and Tina Chiu at the Cumberland Veterinary Clinic for their assistance and correspondence.

This research was funded by the Western College of Veterinary Medicine Companion Animal Health Fund.

Dedication

This thesis is dedicated to my parents, Carolyn and Peng Yong, and to all my friends, who all helped me get to where I am today. None of this would have been possible without your continued support

Table of Contents

List of Abbreviations

ICV – Intracerebroventricular

IP – Intraperitoneal

IRIS – International Renal Interest Society

NUCB2 – Nucleobindin 2

T2DM – Type 2 Diabetes Mellitus

Chapter One: Introduction and Literature Review

This review summarizes the current knowledge surrounding nesfatin-1, a multifunctional protein hormone, and its potential function in lean, overweight, and diabetic cats. The connection between nesfatin-1, glucose homeostasis, and insulin function, as well as the effects of nesfatin-1 on food intake and weight control will be of particular focus. The pathophysiology and current treatment strategies of feline diabetes mellitus will be explored. The current knowledge of central and peripheral tissue expression of nesfatin-1 and the effects of nesfatin-1 on glucose homeostasis will be reviewed. The implications of how these effects could influence our understanding of feline obesity, insulin resistance, and diabetes will be discussed. Finally, the knowledge gaps that are present and how our objectives will address these knowledge gaps will be discussed.

1.1 Obesity and Diabetes Mellitus in Cats

Obesity and obesity-related diseases are an increasing problem in domestic cat populations, following similar trends with human type 2 diabetes mellitus (T2DM). The prevalence of feline diabetes mellitus varies, but is reported to be between 0.25-1%, up from 0.08% from an initial 1970 study [1]. Approximately 90% of feline diabetes cases are of a similar pathophysiological mechanism to T2DM in humans [1], with the major similarity being the presence of insulin resistance, though additional pathological changes seen in humans, such as atherosclerosis and hypertension, are not commonly seen in cats [2]. As insulin resistance and irregular insulin secretion progresses [2,3], persistent hyperglycemia results in a cycle of decreased ß-cell function, continued hyperglycemia and further ß-cell dysfunction [4]. Cats with diabetes are approximately six times less sensitive to insulin than healthy controls [1], though cats are also thought to be able to tolerate insulin resistance and maintain fasting and post-prandial normoglycemia for years before clinical illness manifests [2]. This tolerance is primarily achieved through a reduction in hepatic glucose production [2].

Although diabetes is well described in cats, there are still deficiencies related to both the diagnosis and management of the condition. Treatment of diabetic cats generally involves the administration of exogenous insulin injections and a high protein, low carbohydrate diet, as these

diets are associated with reduced post prandial glucose concentrations and insulin secretion [5]. However, treatment requires owners to administer these injections along with close glucose monitoring at home and/or frequent veterinary visits, requiring significant investments in time, money, and effort. This is not always achievable, and it is estimated around 30% of cats are euthanized within a year of the initial diagnosis [4]. The ideal clinical outcome is for a diabetic cat to enter diabetic remission with treatment, which has been defined as the maintenance of normoglycemia without any need for exogenous insulin for at least four weeks [4]. However, remission rates are variable. In some studies remission rates reach around 20 to 40% [6], while others have seen remission rates of over 80% [7,8]. Such a high remission rate would indicate a favourable prognosis; however, studies achieving high rates of remission were conducted in a veterinary teaching hospital and are likely not representative of the general owned cat population. These remission rates were achieved with rigorous glycemic control, with glucose levels being measured up to five times per day [9], something that is not feasible for many owners.

Another issue is that there is currently no reliable marker to help identify feline diabetics that have a higher chance of achieving remission [4], or to identify the presence of insulin resistance without clinical diabetes i.e., prediabetics [2]. For example, tests that are used in humans to demonstrate insulin resistance, such as the intravenous glucose tolerance test, do not accurately reflect either glucose homeostasis in lean healthy cats or the presence of insulin resistance in obese cats [2]. A reliable assay to measure feline endogenous insulin is also not widely available [2], though an assay designed for feline and human insulin has been validated for use in cats [10]. Furthermore, endogenous insulin concentrations in cats show high levels of intra and inter-individual biological variability [11]. Therefore, a single insulin measurement may not be representative of overall insulin secretory capabilities in cats. A marker that could detect insulin resistance, especially prior to the onset of diabetes mellitus, would provide benefits in both scenarios. If insulin resistance is identified before diabetes occurs, treatment may be less intensive. For example, humans with prediabetes are generally managed with non-pharmacological methods, including weight loss, dietary alterations, and physical activity [12]. Identifying this state in cats might avoid the need for exogenous insulin or rigorous glucose monitoring. Alternatively, if owners could know before beginning treatment that their cats had a high chance of remission, this would provide added motivation to maintain strict glycemic

control, as there would be a higher chance of being able to discontinue treatment. Currently, factors associated with a higher chance of remission include the achievement of rapid glycemic control, administration of glargine insulin twice daily, less severe hyperglycemia at presentation, the absences of hypercholesterolemia at diagnosis, and a shorter duration of diabetes before diagnosis [4,7]. However, these indicators can be unreliable, and in the case of duration of diabetes before diagnosis, difficult to ascertain. Furthermore, the achievement of rapid glycemic control is only able to be measured after treatment is started. If a marker existed that predicted if a cat was likely to achieve rapid glycemic control before starting treatment, as mentioned previously this could provide important additional motivation. Nesfatin-1 has potential as a marker to identify these states in cats.

1.2 Nesfatin-1: Initial Discovery, Central Expression, and Effects on Food Intake

Nesfatin-1 is a recently discovered multifunctional protein hormone, with much of the research related to where it is expressed within the brain, the pathways through which its actions are exerted, and its effects on the modulation of food intake. Nesfatin-1 was initially described in 2006 in a study of rats [13]. It is derived from the precursor nucleobindin 2 (NUCB2), and processed into three separate fractions, nesfatin-1, nesfatin-2, and nesfatin-3, with the functions of nesfatin-2 and 3 currently unknown [13]. Many antibodies used to identify nesfatin-1 cannot distinguish between NUCB2 and the cleaved nesfatin-1 protein, so they are often described together [14]. Nesfatin-1 was identified in areas of the brain in rats (arcuate nucleus, paraventricular nucleus, supraoptic nucleus, lateral hypothalamic area, solitary tract nucleus) that are typically associated with the regulation of food intake [13].

Nesfatin-1 appears to exert its effects though a variety of signalling pathways typically associated with food intake, and these pathways also give rise to additional functions of nesfatin-1. Nesfatin-1 is considered to act through melanocortin signalling in the hypothalamus in rats [13]. This pathway involves the processing of pro-opiomelanocortin (POMC) into alpha-melanocyte stimulating hormone (a-MSH), with POMC expressing neurons located in the hypothalamus and brainstem possessing anorexigenic activity [15]. In pigs, this melanocortin pathway is thought to regulate appetite, growth and adiposity [16]. In support of this,

melanocortin antagonists were found to eliminate the effects of nesfatin-1 on food intake [13]. Similarly, blocking corticotropin releasing factor (CRF) pathways removed the inhibitory effects of nesfatin-1 [17], suggesting nesfatin-1 may act through both melanocortin and CRF pathways to exert its anorexigenic effects. Additional regulation likely occurs through oxytocin, as oxytocin antagonists have been found to suppress the effects of both nesfatin-1 and a-MSH when administered centrally [18].

In addition to its central effects, there is also evidence to suggest that nesfatin-1 acts via a combined central and peripheral route to control food intake, potentially involving the Gut-Hypothalamic-Pituitary axis. Nesfatin-1 associated neurons may be activated by cholecystokinin (CCK), a gut peptide that may act as an endocrine messenger [19] and is associated with satiety signalling [17]. In an *in vitro* mouse model, nesfatin-1 was shown to activate neurons isolated from vagal afferent ganglia, which are also responsive to CCK [20]. Furthermore, gastric acid secretion can be inhibited by central administration of nesfatin-1, via a mechanism that involves vagal efferent nerves [21], and nesfatin-1 is released from the synaptic endings of vagal neurons, suggesting a role in gastrointestinal secretory and motor activity [22]. Nesfatin-1 expressing neurons projecting from the dorsal motor nucleus of the vagus nerve also project to the pancreas and the stomach, which suggests this pathway may have an additional function related to central glucose and insulin signalling [23]. Lastly, nesfatin-1 is able to cross the blood brain barrier via a non-saturable mechanism, with preferential uptake occurring in the hypothalamus [24], which further implies the ability of peripheral nesfatin-1 to affect central nesfatin-1 functions, and vice versa.

Given the areas of centrally expressed nesfatin-1, much of the research has focused on the effects of centrally administered nesfatin-1 in relation to food intake, satiety, and weight loss. Central administration of nesfatin-1 has been found to consistently reduce food intake. Acute intracerebroventricular (ICV) administration of purified nesfatin-1 (5 pmol) decreased food intake for approximately six hours, and chronic ICV administration (10 days) reduced both food intake and body weight gain in rats [13]. Blockage of nesfatin-1 activity then corresponded to an increase in food intake [13]. Similar effects were also demonstrated in pigs [16]. This reduction in food intake occurred in part due to reduced gastric emptying and prolonged gastrointestinal transit time [17,25]. This reduction in motility is one mechanism through which nesfatin-1

induces satiety. This effect is considered to be dose dependant, as higher doses of ICV nesfatin-1 in mice (3 ug) suppressed food intake for at least 12 hours [26], and ICV administration of a dose five times higher than the study by Oh-I (25 pmol/L vs 5 pmol/L) affected food intake for over 48 hours. This higher dose reduced meal size and meal frequency in the mice [26]. Furthermore, fasting reduces NUCB2 mRNA expression in the brain and the stomach [27,28], suggesting that NUCB2/nesfatin-1 levels decrease with prolonged fasting, as no satiety signal is required, and increases again after re-feeding.

1.3 Nesfatin-1 Expression in Peripheral Tissues

In addition to characterization of the central effects of NUCB2/nesfatin-1, expression in peripheral tissues has also been described, with the distribution reflecting its role in various metabolic functions. Nesfatin-1 has been described in the stomach of many different species [28–31], and the stomach is considered to be the major source of circulating nesfatin-1 [14]. Gastric expression of NUCB2/nesfatin-1 mRNA in rats was found to be 10 times higher than in the brain [28]. Furthermore, pro-hormone cleavage enzymes are abundant in gastric mucosa [32], which could also point towards the stomach being the site of both NUCB2 production and processing into nesfatin-1 protein [33]. Nesfatin-1 is also co-expressed with ghrelin within ghrelin producing X/A like endocrine cells and stored within separate populations of intra-cytoplasmic vesicles [28]. These cells have significant roles in appetite control and gastrointestinal motility, two key functions of nesfatin-1. Ghrelin opposes nesfatin-1 by increasing food intake, suggesting that these cells play an important role in food regulation, with differential release of nesfatin-1 and ghrelin depending on the nutritional status of the animal. This further suggests integration between gastric nesfatin-1 expression and central control of food intake.

While the stomach is considered to be the major source of nesfatin-1, several other sites that express nesfatin-1 have been identified. Other gastrointestinal tissues that have been shown to express NUCB2/nesfatin-1 mRNA or nesfatin-1 protein include the pancreas (in ß-cells co-localized with insulin), the small and large intestine in dogs and pigs [31,34,35], and the gut of fish [36]. Additional sites of nesfatin-1 expression include adipose tissue, cardiomyocytes and reproductive tissues [14], and other tissues i.e., liver, adipose tissue, skeletal muscle are affected

by nesfatin-1 [37]. This suggests nesfatin-1 has a pleiotropic action depending on the target tissue, which will be further discussed below. Species differences in the tissue expression of nesfatin-1 likely exist [28,31,35,38], though it can be difficult to determine if these differences are solely due to the species studied, or whether analytical differences play a role e.g., identifying nesfatin-1 protein with western blot or immunoreactivity.

1.4 Potential Differences in Nesfatin-1 Expression in Cats

These potential species differences in peripheral NUCB2/nesfatin-1 expression may be of particular importance in cats given their different metabolic functions compared to other species that are commonly used to study nesfatin-1. While nesfatin-1 has been underexplored in cats, one study has looked into feline nesfatin-1 expression in comparison to other species [31]. In this study, NUCB2/nesfatin-1 mRNA and protein were identified in the stomach of a cat, a dog and a pig [31]. Interestingly, on analysis of the pancreas, it was found that only NUCB2/nesfatin-1 mRNA was found in the cat pancreas, whereas both mRNA and formed protein were found in the ß-cells of the pancreas in dogs and pigs [31]. It was hypothesized that this difference may be due to differences in digestive physiology and glucose metabolism between carnivorous and omnivorous species. It is also hypothesized that this pancreatic nesfatin-1 may act locally on ß-cells in the pancreas to exert effects on insulin regulation [19]. If cats lack this ability, it could represent a risk factor for the development of insulin resistance or altered insulin function in cats. An alternative explanation is that these samples were collected from lean healthy cats, and the presence of nesfatin-1 mRNA could indicate a functional reserve in the pancreas of cats. If this were the case, then nesfatin-1 protein in the pancreas may only be produced in states where there is impaired insulin function e.g., impaired glucose tolerance or diabetes mellitus, and a difference would be seen in expression among lean healthy cats, overweight or obese cats with insulin resistance, and diabetic cats. However, given that only a single cat was evaluated in this study it is difficult to draw any definitive conclusions, and it is possible that the pattern seen in this one cat is not representative of the species as a whole.

1.5 Effects of Peripheral Nesfatin-1 on Food Intake

Peripherally produced nesfatin-1 may also affect food intake and satiety similar to central nesfatin-1, though the evidence is less conclusive. Several studies have investigated the effects of peripherally administered nesfatin-1 on food intake and energy homeostasis, and results are variable, depending on the dose of nesfatin-1 and duration of administration. For example, intraperitoneal administration of 200 pmol of nesfatin-1 in rats did not affect dark phase food intake [17], and a single high dose (7000 pmol) of nesfatin-1 administered IP in mice did not alter meal size or meal frequency. Conversely, a single IP injection of nesfatin-1 (250 pmol/g) in both lean and obese mice resulted in the acute reduction of dark phase food intake [15]. The reason for this discrepancy in the effects of IP nesfatin-1 on food intake is not immediately clear. Peripheral nesfatin-1 shows greater effects when given over a longer period of time. Administration of 5000 pmol/kg/day nesfatin-1 via osmotic pump over 24 hours reduced food intake in the dark phase in rats [27] and this trend was also seen when nesfatin-1 was administered over seven days. Interestingly, physical activity also increased yet body weight was unaffected in this study [27]. Similar results have been seen in fish [39]. Another study in mice found no effect with long term nesfatin-1 administration; however, the dose administered (60 pmol/day) was significantly lower (Prinz Stengel 2016). The main factors in peripheral nesfatin-1 affecting food intake appear to be the dose administered and especially the duration of administration. The half-life of nesfatin-1 in blood is less than 10 minutes in mice [40] and nesfatin-1 is likely excreted through the renal system [24]. Therefore, longer term administration and higher doses of nesfatin-1 are thought to be required to impact food intake significantly, as single injections are cleared too quickly to have a lasting effect. In most animals, it appears that constant infusions or persistently increased circulating levels of nesfatin-1 are likely required for it to affect target tissues, both in relation to food intake and other functions i.e., glucose metabolism. This is in addition to any local or autocrine effects nesfatin-1 may have. Importantly, these effects are likely independent of leptin [15], implying nesfatin-1 maintains its functionality in cases of obesity and leptin resistance.

While peripheral nesfatin-1 does appear to influence food intake, it is debatable whether these changes can be observed by measuring blood nesfatin-1 concentrations. A rat study previously mentioned discovered that rats fasted for 24 hours had significantly decreased plasma

nesfatin-1 concentrations, and levels returned to baseline after re-feeding [17], suggesting food intake can affect peripheral nesfatin-1. Conversely, in a study of dogs fed a variety of diets, gastric mRNA expression of nesfatin-1 was increased with high protein and high fibre diets while plasma nesfatin-1 concentrations were unchanged [30]. This may again point to nesfatin-1 acting directly through vagal pathways without involving circulating nesfatin-1, and a direct connection between gastric and central nesfatin-1. However, the possible connection between fasting and circulating nesfatin-1 suggests that the time of blood sampling e.g., fasted versus post prandial may be an important variable to consider. Furthermore, the potential effects of dietary manipulation on nesfatin-1 expression may warrant further investigation, especially as high protein diets - diets commonly provided to diabetic cats and for the purposes of weight loss - appear to exert significant effects. It remains to be seen whether these diets could induce changes in peripheral nesfatin-1 in cats and whether the different nutritional requirements of cats compared to dogs would change this relationship between diet and nesfatin-1.

Route of Administration	Species	Physiological Effect
Central		
Intracerebroventricular	Rats	Decreased food intake, weight loss [13,26]
		Decreased gastric emptying, prolonged GI transit time [17,25]
	Rats, Mice	Anxiety like behaviour (high doses) [41]
		Increased ACTH and corticosterone concentrations [29]
	Rats	Decreased hepatic gluconeogenesis, increased peripheral glucose uptake [42]
Peripheral		
Intraperitoneal	Rats, Mice	Decreased food intake [15,27], note other studies did not see reduced food intake with IP nesfatin-1 [17,38]
Intravenous	Mice	Decreased plasma free fatty acid levels [43]
		Decreased plasma cholesterol [44]
		Decreased blood glucose (in hyperglycemic mice only) [45]
Subcutaneous	Mice	Increased serum insulin levels and insulin mRNA expression, increased insulin sensitivity [37]

Table 1.1: Effects of nesfatin-1 depending on the route of administration in experimental laboratory animal models

1.6 Correlations Between Nesfatin-1 and Adiposity

Similar to its anorexigenic effects, nesfatin-1 may have implications in obesity and adiposity, indicating a role in overall body metabolism. However, it remains to be seen whether these findings are applicable to cats. In diabetic mice, intravenous (IV) nesfatin-1 administration over six days improved utilization of fatty acids, and reduced plasma free fatty acid levels [43], indicating a link between nesfatin-1 and lipid metabolism. Chronic administration was also found to reduce plasma cholesterol [44]. A study in humans found plasma nesfatin-1 was positively correlated with Body Mass Index (BMI) and levels did not appear to plateau as BMI

increased [46]. Similarly, T2DM patients undergoing bariatric surgery had decreased nesfatin-1 levels that were correlated with decreased BMI [29]. In this context, nesfatin-1 appears to display similar functions to adiponectin. Adiponectin is an adipokine implicated in obesity in cats and dogs, with functions similar to some of those seen with nesfatin-1 i.e., increased insulin sensitivity and peripheral glucose uptake [47]. In humans, adiponectin improves insulin sensitivity via insulin signalling in skeletal muscle and reduced hepatic gluconeogenesis [48]. Given the similar effects of adiponectin and nesfatin-1, altered nesfatin-1 expression is likely implicated (either as a cause or a response to) in the development of obesity and lipid disorders, in addition to its effects on glucose metabolism. These similarities also point towards nesfatin-1 having potential as a marker or even as a treatment of obesity and obesity related disorders, especially given its leptin-independent mode of action.

The role of adiponectin in diabetes and obesity in humans and cats has been investigated. Interestingly, adiponectin levels were decreased in obese cats compared with lean cats and were lower still in diabetic cats [49]. This pattern of expression was also documented in humans. Plasma adiponectin concentrations in humans generally decrease as body weight increases, and adiponectin levels also appear to decrease as the degree of insulin resistance increases [50]. Conversely, despite their similar functions, the opposite association has been demonstrated with nesfatin-1 in humans, where plasma nesfatin-1 concentrations increase as body weight and insulin resistance increase. The effects of nesfatin-1 on reducing cholesterol may also provide additional use in the diagnosis and management of feline diabetes, as hypercholesterolemia has been described as a negative prognostic indicator for diabetic remission in cats [8].

	Functions in Relation to Glucose Homeostasis	Plasma Concentrations in Obese Humans	Plasma Concentrations in Diabetic Humans
Nesfatin-1	Improved insulin sensitivity in insulin sensitive cells [37] Increased insulin secretion from pancreatic ß-cells [51] Reduced hepatic gluconeogenesis and improved peripheral glucose uptake (central administration) [42]	Increased [29,46]	Increased* [52–54] *Only increased in newly diagnosed or untreated diabetics
Adiponectin	Improved insulin sensitivity in skeletal muscle [48] Reduced hepatic gluconeogenesis [48]	Decreased (Weyer *et al.*, 2001)	Decreased [49,50]

Table 1.2: Comparisons between the functions of nesfatin-1 and adiponectin in relation to glucose homeostasis, and differences in plasma nesfatin-1 and adiponectin concentrations in obese and diabetic humans in comparison to lean healthy controls

1.7 Other Clinical Factors That May Influence Nesfatin-1 Expression

Nesfatin-1 likely affects a wide variety of organ systems, and while these effects are not the major focus of this study, they must be considered, especially in the selection of study subjects and identification of other variables that may influence study results. Nesfatin-1 levels were found to be increased in hypertensive human patients who were confirmed to be free of diabetes or impaired glucose tolerance [55]. Conversely, circulating nesfatin-1 levels in humans are significantly decreased with other forms of cardiovascular disease, namely carotid artery stenosis [56] and acute myocardial infarction [57]. Nesfatin-1 is also considered to have cardioprotective effects in mammals [58]. While these potential effects of nesfatin-1 should not impact diabetic cats directly, as hypertension is not a common symptom associated with feline diabetes [2], cats that have concurrent cardiac disease or other diseases affecting blood pressure e.g., advanced renal disease and hyperthyroidism, may have unpredictably altered nesfatin-1

concentrations. Additionally, central nesfatin-1 has been found to induce anxiety-like behaviours in rats [41], although higher doses are required to induce anxiety behaviours than are needed to alter food intake [59]. Studies in humans have also shown elevated nesfatin-1 in patients with depression, with levels positively correlated with the severity of depression [60]. However, nesfatin-1 also varies with both the type of mental illness present and sex of the study subjects [19]. These relationships may be related to the pathways through which nesfatin-1 can act, as ICV nesfatin-1 can increase plasma Adrenocorticotropic Hormone (ACTH) and corticosterone concentrations in rats, likely through interactions with CRF, the hypothalamic-pituitary-axis and the sympathetic nervous system [29,54]. This interaction between nesfatin-1 and the hypothalamic pituitary axis is unsurprising as, generally, food intake, stress, and anxiety are closely related [14]. Again, while this interaction should not specifically affect diabetic patients, if they have any diagnosed or clinically suspected anxiety or behavioural disorders, nesfatin-1 expression may be altered.

1.8 Correlations Between Nesfatin-1 and Glucose Homeostasis

One of the important functions of nesfatin-1 is its insulinotropic effects on glucose homeostasis. Previous research has found that nesfatin-1 can directly stimulate insulin secretion from ß-cells, primarily in hyperglycemic states. For example, *in vitro* studies on isolated mouse pancreatic islet cells found that nesfatin-1 increased insulin secretion in a dose- dependent manner, and this effect was significantly greater in cells that were exposed to high glucose concentrations [51]. Furthermore, higher nesfatin-1 concentrations than what are typically seen in lean, healthy subjects are required to stimulate insulin secretion from pancreatic islets in mice [19], and IV injections of nesfatin-1 significantly decreased blood glucose in hyperglycemic mice only [45]. In this mouse study, blood levels of insulin were largely unchanged in the short term [45]. However, nesfatin-1 administered to a type 1 diabetes mellitus mouse model did not affect blood glucose levels, indicating insulin is required for nesfatin-1 to function [45]. Other studies did demonstrate increased serum insulin levels after chronic sub-cutaneous nesfatin-1 administration, with *in vitro* models showing increased insulin mRNA expression [37]. Together, these findings imply that nesfatin-1 predominantly influences glucose homeostasis when glucose homeostasis is impaired. Nesfatin-1 may act by both increasing insulin sensitivity and insulin

secretion, supporting the idea of nesfatin-1 acting as a mechanism to try and maintain insulin function when insulin resistance is present.

Aside from nesfatin-1's direct effects on the pancreas, it also has additional effects on peripheral tissues in relation to glucose homeostasis. In both *in vitro* and *in vivo* models, the effects of insulin and nesfatin-1 on AKT phosphorylation, a marker of peripheral insulin signalling in islet cells, liver, skeletal muscle and adipose tissue, were studied [37]. Administration of peripheral nesfatin-1 increased AKT phosphorylation in all tissues, in both control mice and mice fed a high fat diets [37]. This increased phosphorylation suggests that nesfatin-1 improves insulin sensitivity in peripheral tissues and pancreatic islet cells, and that this may occur in both lean and obese animals. The administration of nesfatin-1 also increased the expression of glucose transporter 4 in muscle and adipose tissue. Furthermore, expression of NUCB2/nesfatin-1 mRNA and protein in muscle and adipose tissue was increased in human T2DM patients compared with healthy controls [53]. These findings indicate a role for nesfatin-1 in compensating for altered insulin function by improving peripheral uptake of glucose.

There is also evidence that central nesfatin-1 may have an influence on overall glucose homeostasis, and this finding may be of particular relevance in cats. In one study, an ICV dose of nesfatin-1 was administered in rats with insulin resistance, and it was found that this dose significantly increased the ability of insulin to suppress hepatic gluconeogenesis and enhance peripheral glucose uptake [61]. This implies a connection between central and peripheral nesfatin-1 on glucose homeostasis, either through a direct neural pathway or via the blood brain barrier. One of the pathological mechanisms in the development of impaired glucose tolerance in humans is the reduced ability of insulin to suppress hepatic gluconeogenesis [12]. This mechanism may be of particular importance in cats, as cats are able to tolerate insulin resistance predominantly through their ability to maintain hepatic insulin sensitivity [2]. If this ability is in part mediated by nesfatin-1, then insulin resistance could potentially be identified by measuring circulating nesfatin-1 in cats.

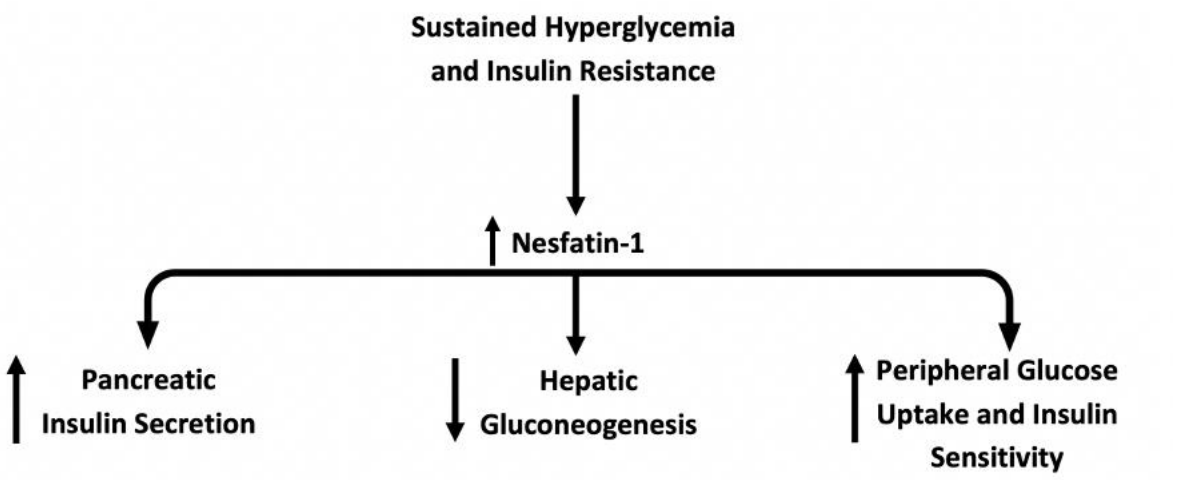


Figure 1.1: Suggested mechanisms by which nesfatin-1 may act to maintain insulin sensitivity and counteract persistent hyperglycemia

1.9 Nesfatin-1 and Diabetes Mellitus in Humans, and the Potential Utility of Nesfatin-1 in Feline Diabetics

Given these effects on glucose homeostasis, nesfatin-1 has been investigated in relation to disorders of glycemic control. Nesfatin-1 levels in human T2DM were inconsistent. Some studies have shown that circulating nesfatin-1 concentrations are lower in T2DM patients compared with healthy controls [33,62], whereas other studies showed the opposite association [52–54]. A meta-analysis [63] revealed that the main factor in these disparate results was whether patients had received diabetic treatment, with the time since diagnosis also being a partial factor. Patients with newly diagnosed untreated T2DM had elevated nesfatin-1 levels compared with controls. However, diabetics that were receiving treatment had lower nesfatin-1 concentrations compared with healthy controls. This decrease in nesfatin-1 may be due to either the resumption of glycemic control and the reversal of glucose toxicity, or the continued loss or dysfunction of pancreatic ß-cells, resulting in decreased insulin secretion and consequently reduced stimulus for nesfatin-1. Nesfatin-1 has also been found to be increased in patients with impaired glucose tolerance (IGT) [52,54], which is analogous to a prediabetic state. In contrast, another study found that nesfatin-1 was decreased in IGT patients [62], though it is unclear if these patients were receiving any other treatments e.g., oral anti-hyperglycemics. These

inconsistent findings make it difficult to predict nesfatin-1 levels in patients that achieve diabetic remission i.e., the resumption of glycemic control without the requirement of exogenous insulin, as these patients undergo treatment, but then at some point are no longer considered diabetic. In cats at least, it is suggested that diabetics that achieve remission still do not have normal ß-cell function and clinically should be considered 'prediabetic' [9]. This residual ß-cell dysfunction suggests that as remission occurs, nesfatin-1 may return to healthy control levels as glycemic control resumes or may stay slightly elevated due to the residual impaired glucose tolerance. In summary, it appears that circulating nesfatin-1 concentrations increases with impaired glucose tolerance and insulin resistance, culminating with T2DM. Then, as T2DM patients receive treatment, and possibly as treatment continues over time, nesfatin-1 levels decrease. This pattern could be related to continued glucose toxicity and ß-cell necrosis over time, which could subsequently lead to reduced nesfatin-1 secretion.

Overall, the changes in nesfatin-1 seen in disorders of glycemic control, and the similarities of feline diabetes mellitus and human T2DM demonstrate the potential utility of circulating nesfatin-1 in feline patients. Specifically, as insulin resistance occurs, nesfatin-1 concentrations may increase as a compensatory mechanism to help maintain insulin sensitivity and normoglycemia. If so, this mechanism indicates that nesfatin-1 could be used as a marker of insulin resistance, both when clinical diabetes mellitus is present, but also to identify a prediabetic state. Identifying insulin resistance before clinical diabetes would be the ideal scenario as treatment is less arduous for the owner. It may also be of particular interest in cats given their compensatory abilities and how long they tolerate insulin resistance [2]. This prolonged time frame would theoretically give a larger clinical window for insulin resistance to be identified and managed. As clinical diabetes develops, nesfatin-1 may have additional use in monitoring the response to therapy, or possibly helping to predict the chances of remission being achieved. Given the findings of nesfatin-1 in relation to long term human diabetics [33,62], the use of nesfatin-1 in the long-term monitoring of diabetes may be of less benefit, due to the continued ß-cell dysfunction and/or necrosis. Given the effects of nesfatin-1 in relation to its effects on lipid metabolism, cholesterol, food intake, and glycemic control, nesfatin-1 may even have use as a therapeutic treatment in the management of obesity, diabetes, and other metabolic disorders.

1.10 Summary and Research Objectives

Despite the connections between nesfatin-1, diabetes, obesity, adiposity, and food intake, and the similarities between feline diabetes and human T2DM, nesfatin-1 has been underexplored in cats. At this time there is only one study that has investigated nesfatin-1 in cats [31]; This study evaluated nesfatin-1 expression in the stomach and pancreas of a single cat, and the plasma of eight healthy cats. While it is useful to have a small amount of baseline data, as well as the knowledge that nesfatin-1 is detectable in feline blood [31], there is room to expand our understanding. No research has been conducted into nesfatin-1 expression in overweight or diabetic cats, and this knowledge gap is what this thesis aims to evaluate. Chapter one will investigate tissue expression of nesfatin-1 mRNA in gastro-intestinal and peripheral tissue, and comparisons made between lean, overweight, and diabetic cats. Chapter two will then evaluate and characterize plasma nesfatin-1 concentrations between these groups, as well as investigating how treatment of diabetic patients impacts plasma nesfatin-1 concentrations. The potential changes in nesfatin-1 expression among groups will provide further information as to whether nesfatin-1 changes with metabolic disorders in cats in similar ways to other species, namely humans and laboratory animals.

Given the similarities, in particular the development of insulin resistance, between feline diabetes and human T2DM, we hypothesize that nesfatin-1 will play a role in the compensation for insulin resistance and altered glucose homeostasis in cats. However, the specialized metabolic and nutritional physiology found in cats adds a potential unpredictable variable. For plasma nesfatin-1 concentrations, we hypothesize that newly diagnosed diabetic cats will have significantly higher nesfatin-1 levels compared with lean and overweight healthy cats, and that levels will decrease as cats undergo treatment and glycemic control improves. Conversely, an additional effect may be seen in patients that undergo remission during the study. These patients may have lower nesfatin-1 at presentation compared with diabetic cats that do not achieve remission, which would reflect comparatively better glycemic control at the start of treatment. Overweight cats may have higher nesfatin-1 levels than lean healthy controls, particularly if these overweight cats have some degree of insulin resistance. We also hypothesize that we will see similar changes in nesfatin-1 tissue mRNA expression among lean, overweight, and diabetic cats. Expression may be higher in diabetic and overweight cats compared with lean healthy cats,

owing to increased synthesis of nesfatin-1 as a compensatory mechanism for the presence of insulin resistance.

Chapter Two: Evaluation of NUCB2/ Nesfatin-1 mRNA Tissue Expression in Lean, Overweight, and Diabetic Cats

2.1 Abstract

Background: Rates of obesity and diabetes mellitus are increasing in domestic cats. Nesfatin-1 is a protein hormone implicated in controlling food intake and maintaining glycemic control. The role of nesfatin-1 in the pathophysiology of obesity and diabetes mellitus in cats has not been evaluated. This study aimed to characterize NUCB2/nesfatin-1 mRNA expression in feline gastric, pancreatic, abdominal adipose, and skeletal muscle tissue, and to evaluate how tissue nesfatin-1 expression differs between lean, overweight, and diabetic cats.

Methods: Case control study. Four lean, three obese, and five diabetic cats were sampled. RNA was extracted from pancreas, stomach, abdominal fat, and skeletal muscle, and a one-step RT-PCR was performed. Relative NUCB2/nesfatin-1 expression was assessed by the comparative $2^{-\Delta\Delta Ct}$ method.

Results: NUCB2/nesfatin-1 mRNA expression was highest in the pancreas and there was a wide variance in tissue expression in all cats. Expression tended to be marginally decreased in pancreatic tissue of overweight cats in comparison to lean cats. NUCB2/nesfatin-1 expression in the gastric and pancreatic tissue of diabetic cats showed a biphasic pattern. Two diabetic cats had similar expression patterns to lean cats, while three diabetic cats had decreased expression compared to lean cats. This biphasic expression was unrelated to the body condition or treatment status of these diabetic cats. No patterns of expression were identified in adipose and muscle tissue.

Conclusions: The pancreas had the highest levels of NUCB2/nesfatin-1 mRNA expression in cats. Changes in expression in diabetic cats were most pronounced in the stomach and pancreas. The biphasic pattern of expression in diabetic cats may reflect differences in pancreatic islet health and function in these cats. The clinicopathological implications of these changes need to be investigated further. Future studies may involve assessing changes in nesfatin-1 mRNA expression in conjunction with changes in nesfatin-1 tissue protein expression and circulating nesfatin-1 concentrations.

2.2 Introduction

The prevalence of obesity and obesity-related diabetes mellitus is increasing in both humans and cats [1]. Feline diabetes shares many clinicopathological features with human type 2 diabetes mellitus (T2DM), such as middle age, obesity, and peripheral insulin resistance [2,47]. It is considered that approximately 90% of feline diabetes cases share similar pathophysiological mechanisms to T2DM in humans, namely the development of insulin resistance [1]. Furthermore, cats with diabetes are approximately six times less sensitive to insulin than healthy controls [1], and cats are able to tolerate insulin resistance for years before clinical illness manifests [2].

Nesfatin-1 is a highly conserved, multifunctional protein hormone derived from the nucleobindin 2 (NUCB2) gene. It is expressed within multiple regions in the brain in rats, particularly in areas involved in feeding regulation and appetite control [13,17]. Nesfatin-1 has been found to exert a wide variety of functions in various species, including control of glucose homeostasis, food intake and adiposity. Intracerebroventricular injection of nesfatin-1 in rats has been shown to reduce gastric emptying [25], food intake, and weight gain [13]. Chronic peripheral administration of nesfatin-1 may also reduce food intake and modulate energy homeostasis in rats [27]. Furthermore, the stomach is considered the main source of circulating nesfatin-1 [14], suggesting a link between dietary composition, weight loss and peripheral nesfatin-1 levels. Intravenous nesfatin-1 administration has been found to increase the expression of enzymes promoting fatty acid oxidation in skeletal muscle [43] and decrease fatty acid synthesis in the liver [38]. In the mouse, nesfatin-1 has insulinotropic effects. It can directly stimulate insulin secretion from pancreatic islet cells in vitro, and augment insulin secretion and glucagon suppression in vivo [51]. Furthermore, higher nesfatin-1 concentrations than what are seen in lean healthy subjects are required to stimulate insulin secretion [19], and IV nesfatin-1 only significantly decreased blood glucose levels in mice that were hyperglycemic [45]. Intravenous administration of nesfatin-1 also helps to normalise serum fatty acid levels and body weight in diabetic mice [14]. Nesfatin-1 has also been shown to increase uptake of glucose in peripheral tissues and improve insulin sensitivity [38]. These effects may reflect a potential diagnostic or therapeutic role of nesfatin-1 in patients with obesity or diabetes mellitus.

Despite the increasing prevalence of obesity and diabetes in cats [1] and the functions of nesfatin-1 in relation to these issues, nesfatin-1 is relatively under explored in this species. In domestic veterinary species, nesfatin-1 expression has been described in the stomach and pancreas of a single dog, pig, and a cat [31], but further research investigating nesfatin-1 expression in cats is lacking. The objective of this study was to further understand the role of nesfatin-1 in the pathophysiology of obesity and diabetes mellitus in cats by evaluating differences in tissue expression of NUCB2/nesfatin-1 mRNA between lean, overweight, and diabetic cats. This research will expand the current knowledge of gastrointestinal nesfatin-1 expression in cats and its potential relationship with glucose metabolism and the development of diabetes mellitus.

2.3 Materials and Methods

2.3.1 Study Design

A case-control study was performed on lean, overweight, and diabetic cats. Cats of any age were eligible for enrolment. Lean cats were required to have a body condition score of 4 or 5/9, and overweight cats a body condition score of greater than 6/9. Diabetic cats were eligible to be recruited regardless of body condition score or treatment status. Cats were excluded from the study if there was any laboratory evidence of severe renal disease (IRIS stage three or four) or hyperthyroidism on a recent biochemistry panel, or from a biochemistry panel run at the time of euthanasia. Cats were also excluded if there was previous clinical evidence of heart disease, or evidence of disseminated or pancreatic neoplasia during sampling. Tissues from four lean cats were donated by a collaborator at the University of Calgary Faculty of Veterinary Medicine (UCVM). Tissues from overweight and diabetic cats were collected for this study and for a separate study investigating expression of other gastrointestinal hormones from cats presenting to the Western College of Veterinary Medicine (WCVM) or the UCVM for euthanasia. Experimental protocols were approved by the University of Saskatchewan Animal Research Ethics Board (AUP 20170106) and the University of Calgary Animal Care and Use Committee (AC20-0023). All protocols were conducted in compliance with each university's ethical guidelines for animal research. No cats were euthanized solely for the purpose of this or the other study, but at the request of owners for other health related issues.

2.3.2 Demographics

Four lean cats, three overweight cats, and five diabetic cats were recruited overall. Of the five diabetic cats that were recruited, two were newly diagnosed i.e., they had not received prior insulin therapy, one cat had received insulin for three weeks before euthanasia, and two cats had received insulin therapy for approximately two months before being euthanized due to severe diabetic ketoacidosis (see supplemental table 2.1). One diabetic cat tested Feline Leukemia Virus (FeLV) positive on an FeLV blood antigen test (SNAP FIV/FeLV Combo; IDEXX, Canada).

2.3.3 Sample Collection

For all cats, euthanasia was performed with IV pentobarbital and tissue collection was performed within 30 minutes following euthanasia. Samples of the gastric body, right limb of the pancreas, abdominal skeletal muscle, and abdominal adipose tissue were procured, immediately snap frozen in liquid nitrogen, and stored at -80°C until RNA extraction. Two of the lean cats did not have abdominal fat available to sample, and one did not have any available pancreatic tissue (all pancreatic tissue had been used in a prior study).

2.3.4 RNA Extraction

RNA extraction was performed using different methods for pancreatic and stomach tissue compared with adipose and muscle tissue, given the nature of the tissues and comparative RNA yields. For pancreas and stomach, tissue was homogenized using RINO RNA lysis tubes (catalogue number NAVYR1-RNA; Froggabio, Canada) in 300 uL of buffer RLT as part of the RNeasy mini (catalogue number 74104; Qiagen, Canada) RNA extraction kit. The remainder of the RNA extraction was performed according to manufacturers' instructions. For muscle and fat tissue, A TRIzol RNA purification kit (catalogue number 12183555, ThermoFisher, Canada) was used given the need for stronger cell lysis capability in order to maximise RNA yield. Tissues were homogenized with RINO RNA lysis tubes in 600 uL of Trizol, and RNA extraction was then performed using the kit according to manufacturers' instructions.

For all samples, RNA purity and concentration were evaluated by spectrophotometry (Nanodrop, ThermoFisher, Canada) using 260 nm/280 nm optical density (OD) ratios. RNA integrity was also evaluated by assessing 18S and 28S Ribosomal RNA bands on agarose gel electrophoresis. A portion of the sample was diluted to a concentration of 50 ng/uL, and genomic DNA was removed by DNase digestion (Invitrogen DNA removal kit, catalogue number AM1906; Fisher Scientific, Canada). The newly diluted sample was again evaluated with OD 260 nm/280 nm ratios using Nanodrop spectrophotometry to ensure RNA quality after DNA digestion. RNA was stored at -80°C until analysis.

2.3.5 RT-PCR Protocol

All samples were run in triplicate with a one-step Real Time PCR kit (Quantifast SYBR Green RT-PCR, catalogue number 204154; Qiagen, Canada) according to manufacturer's instructions. Briefly, a 25 uL reaction mix was made with 12.5 uL of master mix, 6.25 uL of RNase free water, 2 uL of each forward and reverse primer, 2 uL of sample RNA, and 0.25 uL of Reverse Transcriptase mix. A negative reverse template control was performed with each sample (0.25 uL of Reverse Transcriptase mix was replaced with an additional 0.25 uL of RNase free water), along with a non-template control (2 uL of template replaced with 2 uL of RNase free water). Real time cycling conditions were set with an initial reverse transcription of 10 minutes at 50°C, followed by an activation step of 5 minutes at 95°C. A two-step cycling method was then performed, with 35 cycles of denaturation (10 seconds at 95°C) followed by combined annealing and extension (30 seconds at 60°C). Melt curve analysis was performed to ensure specificity of the amplified product, and agarose gel electrophoresis was also performed to confirm the targeted amplicon size. The primers to evaluate NUCB2/nesfatin-1 gene expression and one reference gene, ß-actin, were validated in a previous study [31]. A second reference gene, Ribosomal Protein S7 (RPS7), was selected based on a previous study demonstrating its stability in feline tissues in comparison to other reference genes [64] (see supplemental table 2.2 for primer sequences).

2.3.6 Statistical Analysis

Relative tissue expression was assessed using the comparative $2^{-\Delta\Delta Ct}$ method [65]. Briefly, Ct values were obtained for the three amplified gene products, NUCB2/nesfatin-1, RPS7 and ß-actin. For each tissue, the two reference genes (RPS7 and ß-actin) were averaged, and this combined figure was used to normalize NUCB2 expression. For comparison of NUCB2 expression between the different tissue types, the NUCB2 ΔCt values in the stomach of all lean, overweight, and diabetic cats were averaged. ΔCt values from the remaining tissues were then compared to these averaged stomach values for each group. Normal distribution of these Ct values was assessed by a Shapiro-Wilk Test. Differences between NUCB2 expression in the stomach, pancreas, skeletal muscle, and abdominal adipose were assessed by a Kruskal-Wallis

ANOVA, followed by a Dunn's test with Bonferroni correction. Statistical significance was set at $P < 0.05$. For comparison of tissue expression between lean, overweight, and diabetic cats, the NUCB2 ΔCt values in the stomach, pancreas, abdominal adipose tissue, and skeletal muscle were calculated, and expression from individual tissues were compared to the same averaged stomach value as per previous. No further statistical analysis was performed on these values due to the small sample sizes of each individual group. All graphs were created with GraphPad Prism 8® (GraphPad Software, CA).

2.4 Results

2.4.1 Relative NUCB2/Nesfatin-1 mRNA Expression Between Tissue Types

In all cats, pancreatic mRNA expression of NUCB2/nesfatin-1 was significantly higher than expression in gastric ($P < 0.01$), skeletal muscle ($P < 0.01$), and abdominal adipose ($P = 0.01$) tissue (Figure 2.1). Expression in the other three tissues was similar, with relative mRNA expression slightly lower in skeletal muscle ($P = 0.6$), and slightly higher in abdominal adipose tissue ($P = 0.1$) in comparison to gastric expression, though these differences were not statistically significant. However, these differences in expression were small especially given the moderate to marked increase in pancreatic mRNA expression. The variance in pancreatic expression was also the most extreme, with a greater than 10-fold difference in expression amongst the diabetic cats (low value 11.08, high value 147.03). The variances in expression between gastric (0.43 – 2.22), skeletal muscle (0.32 – 1.8), and abdominal adipose tissue (0.62 – 3.39) were much smaller and showed similar dispersion. No overt pattern of expression was present in the skeletal muscle and abdominal adipose tissue.

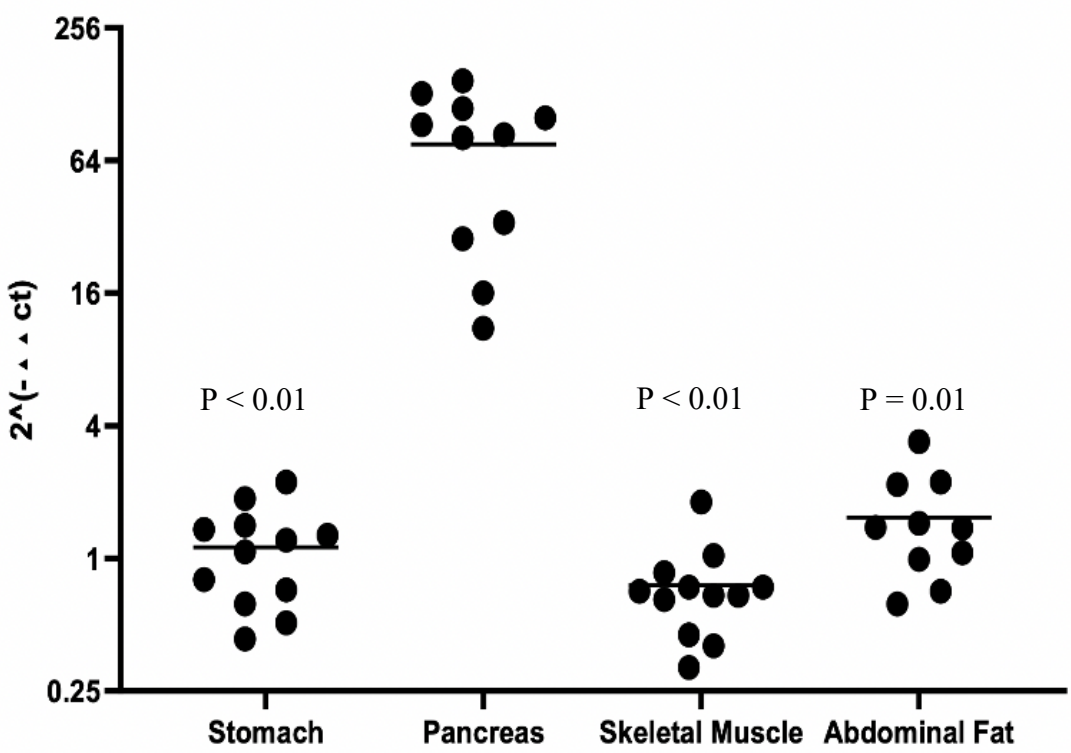


Figure 2.1: NUCB2/nesfatin-1 mRNA expression of gastric (n = 12), pancreatic (n = 11), skeletal muscle (n = 12), and abdominal fat (n = 10) tissue of all lean, overweight, and diabetic cats collected at the Western College of Veterinary Medicine and the University of Calgary Faculty of Veterinary Medicine, relative to the average NUCB2/nesfatin-1 expression of gastric tissue in all cats (n = 12), Kruskal-Wallis ANOVA

2.4.2 Relative NUCB2/Nesfatin-1 mRNA Expression Between Lean, Overweight, and Diabetic Cats

Expression of NUCB2/nesfatin-1 mRNA in gastric tissue, pancreatic tissue, skeletal muscle, and abdominal fat are presented in figures 1.2-1.5. Overall, mRNA expression appeared decreased in the overweight and diabetic groups in comparison to lean cats, particularly in the gastric and pancreatic tissue, though these differences were not statistically significant. However, there is overlap in expression between each of the groups in all the tissues studied. Significant individual variation also exists between cats in all three groups. Two diabetic cats (one untreated and one treated for two to three months) had high levels of NUCB2/nesfatin-1 mRNA expression in their stomach and pancreas, similar to levels seen in lean cats. In contrast, the remaining three diabetic cats (one untreated, one treated for three weeks, and one treated for two months) had much lower NUCB2/nesfatin-1 expression. In this way, the diabetic cats appear to show a

biphasic pattern of expression in pancreatic and gastric tissue. All three overweight cats had lower pancreatic NUCB2/nesfatin-1 expression compared with the lean cats. One overweight cat had particularly low expression, though this cat did not have concurrently low gastric expression. No obvious patterns in expression in the skeletal muscle and abdominal fat were identified.

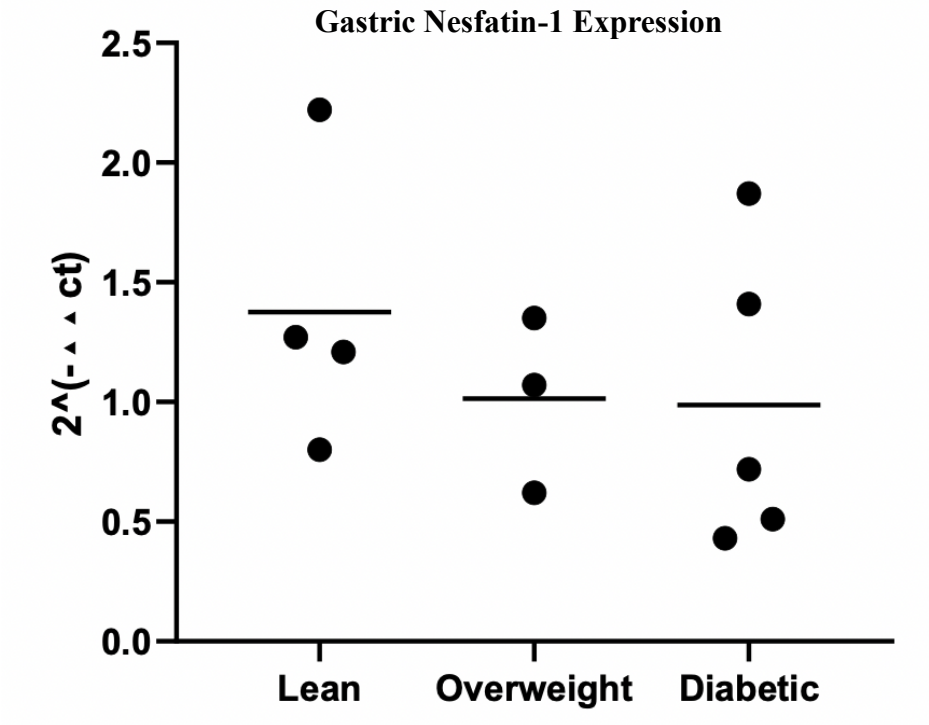


Figure 2.2: NUCB2/nesfatin-1 mRNA expression of gastric tissue in individual lean (n = 4), overweight (n = 3), and diabetic cats (n = 5) collected at the Western College of Veterinary Medicine and the University of Calgary Faculty of Veterinary Medicine, relative to the average NUCB2/nesfatin-1 expression of gastric tissue in all cats (n = 12)

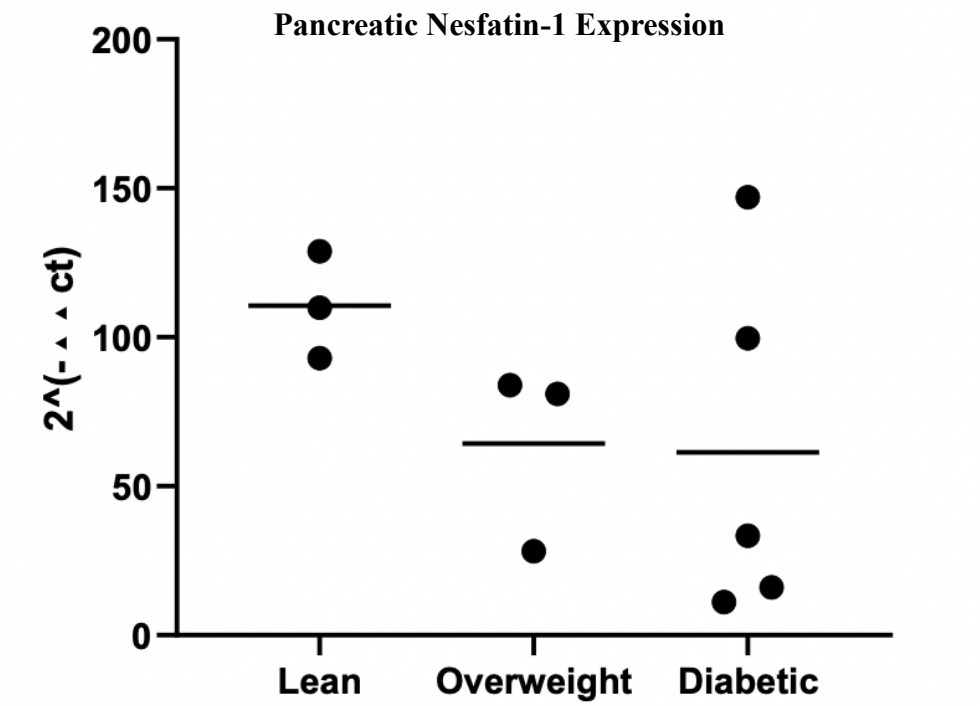


Figure 2.3: NUCB2/nesfatin-1 mRNA expression of pancreatic tissue in individual lean (n = 3), overweight (n = 3), and diabetic cats (n = 5) collected at the Western College of Veterinary Medicine and the University of Calgary Faculty of Veterinary Medicine, relative to the average NUCB2/nesfatin-1 expression of gastric tissue in all cats (n = 12)

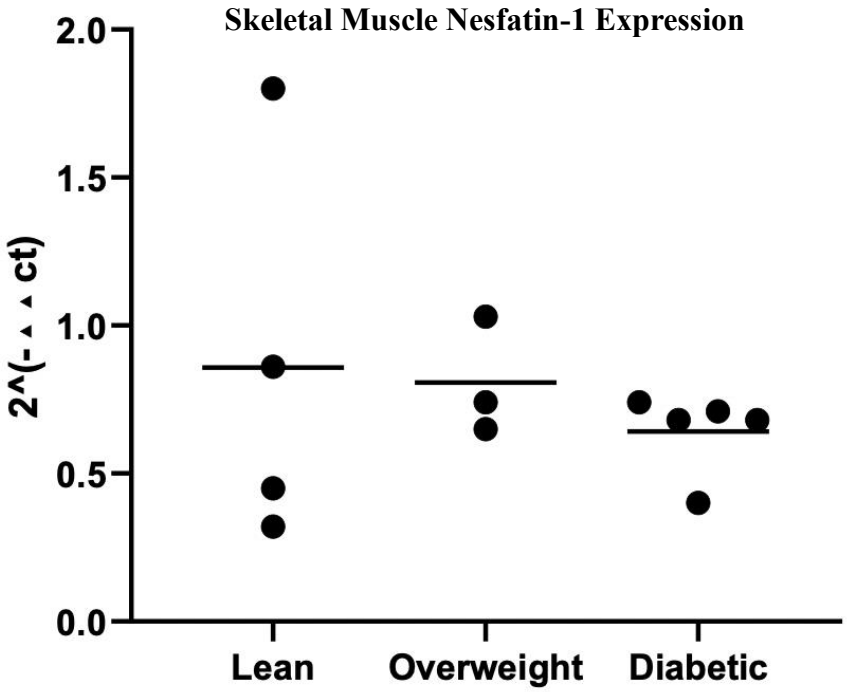


Figure 2.4: NUCB2/nesfatin-1 mRNA expression of skeletal muscle tissue in individual lean (n = 4), overweight (n = 3), and diabetic cats (n = 5) collected at the Western College of Veterinary Medicine and the University of Calgary Faculty of Veterinary Medicine, relative to the average NUCB2/nesfatin-1 expression of gastric tissue in all cats (n = 12)

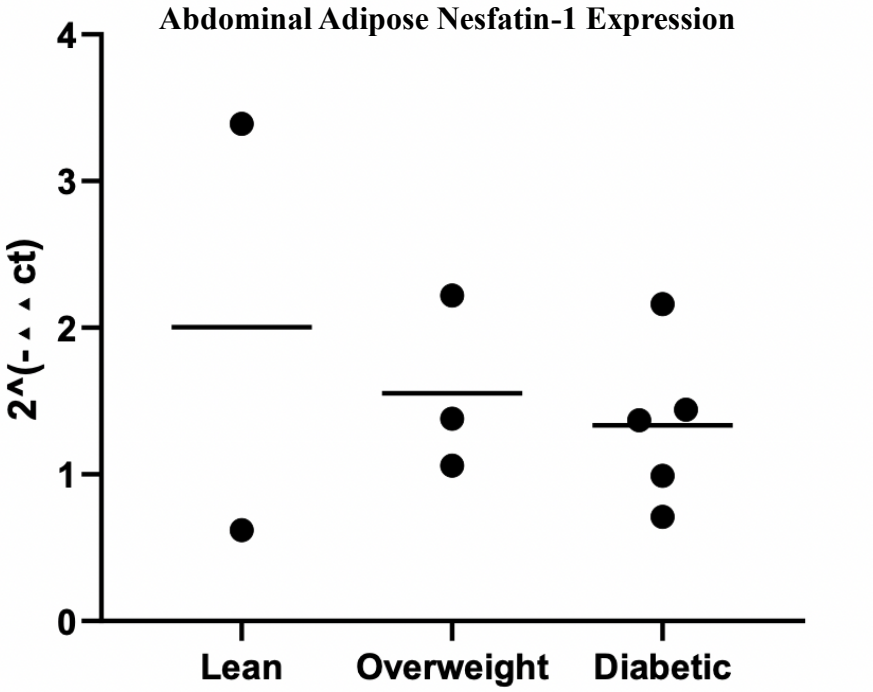


Figure 2.5: NUCB2/nesfatin-1 mRNA expression of abdominal adipose tissue in individual lean (n = 2), overweight (n = 3), and diabetic cats (n = 5) collected at the Western College of Veterinary Medicine and the University of Calgary Faculty of Veterinary Medicine, relative to the average NUCB2/nesfatin-1 expression of gastric tissue in all cats (n = 12)

2.5 Discussion

The results of this study definitively demonstrate that NUCB2/nesfatin-1 mRNA is expressed in feline stomach, pancreas, skeletal muscle, and abdominal adipose tissue. mRNA expression is tissue dependent, with the highest expression occurring in the pancreas. This study also revealed that NUCB2/nesfatin-1 may be differentially expressed in tissues from lean, overweight, and diabetic cats, although significant overlap exists between the study groups. Overall, tissue expression was marginally lower in overweight cats compared with lean cats, whereas diabetic cats displayed a biphasic expression pattern in gastric and pancreatic tissue.

2.5.1 NUCB2/Nesfatin-1 mRNA Expression Between Tissues

In all cats, NUCB2/nesfatin-1 mRNA expression was significantly higher in pancreatic tissue compared to gastric, skeletal muscle, and abdominal adipose tissue. The high pancreatic expression was surprising, given that the gastric glandular mucosa is considered to be the predominant source of circulating nesfatin-1 in rats [28] and thus the stomach was expected to have the highest level of NUCB2/nesfatin-1 expression in cats as well. There are several possible explanations for this result. One possibility is that there are species-specific differences in nesfatin-1 expression. The previous studies documenting gastric mucosa as being the primary source of circulating nesfatin-1 were performed in rats, so this may not accurately reflect expression in cats. Secondly, it is possible some or all the pancreatic mRNA expressed is not converted into formed nesfatin-1 protein, and, therefore, will not be a significant contributor to circulating nesfatin-1 concentrations. Discordance between NUCB2/nesfatin-1 mRNA and protein expression has been documented in cats previously [31], and a study in dogs also suggests that changes in mRNA expression may not necessarily reflect changes in circulating nesfatin-1 concentrations [30]. Another consideration is that the relative expression between tissues may not reflect total mRNA and protein production in the individual tissues. While relative gastric expression is lower, the total amount of mRNA and therefore to total amount of protein produced may still be higher in the stomach, and, therefore, still represent the major site of peripheral nesfatin-1 production. Furthermore, it is unknown if production of nesfatin-1 is uniform or compartmentalized throughout the stomach, therefore sampling a small area of the

gastric body mucosa could have inadvertently missed more productive areas of mucosa. However, this scenario is considered less likely given the magnitude of the difference between pancreatic and gastric expression.

Lastly, the role of pancreatic NUCB2/nesfatin-1 expression must be considered. *In vitro* studies have shown that nesfatin-1 directly increases insulin secretion from pancreatic islet cells exposed to hyperglycemic conditions [51], and a local autocrine mode of action for pancreatic nesfatin-1 has been hypothesized in rats [66]. As such, NUCB2/nesfatin-1 expressed within the feline pancreas may act in a similar manner without significantly contributing to circulating nesfatin-1 concentrations.

2.5.2 NUCB2/Nesfatin-1 mRNA Expression in Diabetic Cats

The most intriguing changes seen were the NUCB2/nesfatin-1 expression in gastric and pancreatic tissue in the five diabetic cats, who showed two relatively distinct patterns of expression, with two diabetic cats expressing pancreatic and gastric NUCB2/nesfatin-1 at similar levels to lean healthy cats, and three diabetic cats have decreased pancreatic and gastric expression. The possible prognostic significance of this biphasic expression needs to be investigated further e.g., with an increased number of study subjects. Interestingly, the diabetic cats with higher expression both had lean body condition scores (4/9), although the three cats with low expression were of variable body condition (6/9, 6/9, and 1/9 respectively). Given this variability, it is unlikely that differences in condition score or adiposity had a major effect on the differences in mRNA expression in the diabetic cats, though it cannot be excluded. Similarly, the two diabetic cats with higher NUCB2/nesfatin-1 expression were spayed females, while two of the three diabetic cats with low expression were castrated males. This could suggest some degree of sexual dimorphism between NUCB2/nesftin-1 expression, though this is of questionable significance given the small sample size.

Another possibility is that the individual variance in feline pancreatic expression of NUCB2/nesfatin-1 may be an indicator of islet health or pancreatic ß-cell function. Previous research has found that the anti-hyperglycemic effects of nesfatin-1 are time, dose, and insulin-dependent [45]. Furthermore, pancreatic nesfatin-1 protein expression was found to be lower in a

type 2 diabetic rat model compared with lean healthy rats [66]. It follows that pancreatic expression of NUCB2/nesfatin-1 mRNA may be correlated with insulin production and islet health. Healthy lean cats may have comparatively better islet health and insulin secretory capabilities and, therefore, higher NUCB2/nesfatin-1 expression. In diabetic cats, the progressive islet cell loss or continued glucose toxicity and resulting decreased insulin secretion may potentially lead to decreased NUCB2/nesfatin-1 expression. Similarly, this could also explain the biphasic expression seen in the diabetic cats. The three diabetic cats with reduced NUCB2/nesfatin-1 mRNA expression may have comparatively poorer islet cell function in comparison to the two diabetic cats that had gastric and pancreatic mRNA expression similar to the lean healthy cats. If this is the case, and these two cats did have comparatively better islet cell health, it would also suggest these two cats may also have been more responsive to diabetic treatment.

Alternatively, nesfatin-1 has been found to increase insulin secretion in the presence of hyperglycemia [51], and higher nesfatin-1 concentrations than those in lean subjects are required to stimulate insulin secretion in mice [19]. These findings could suggest the opposite association, that increased pancreatic NUCB2/nesfatin-1 expression theoretically represents a compensatory attempt to maintain insulin secretion in animals with poor islet cell health and diminished insulin secreting capabilities i.e., diabetics. However, this scenario may be considered less likely as if this were the case, lean healthy cats would be expected to have low NUCB2/nesfatin-1 expression, with expression increased in diabetic cats with islet cell damage. Overall, what these changes in NUCB2/nesfatin-1 mRNA expression mean in regard to prognostic significance and the different expression patterns in diabetic cats requires further investigation.

2.5.3 NUCB2/Nesfatin-1 mRNA Expression Between Lean and Overweight Cats

In overweight cats, the overall trend in NUCB2/nesfatin-1 tissue expression, particularly in pancreatic and gastric tissue, was that mRNA expression appeared to be marginally decreased in comparison to lean cats. This trend was unexpected, as previous research in humans has found that plasma nesfatin-1 levels and gastric nesfatin-1 protein expression are positively correlated with body mass index [29,46]. This increased expression in overweight humans is hypothesized

to be a compensatory response by nesfatin-1 to increase anorexigenic signalling [29]. The reason for this discrepancy between our findings and the findings in humans is unclear. It may reflect that cats do not express nesfatin-1 in the same ways as other species as suggested by a previous study [31], or that tissue mRNA or protein expression may not necessarily correlate with circulating nesfatin-1 concentrations [30,66]. Speculatively, given that obesity and insulin resistance are closely linked, these findings could indicate differences in pancreatic islet health, similar to the diabetic cats. If the overweight cats have insulin resistance and poor islet cell function, then this could be reflected by a decrease in NUCB2/nesfatin-1. In this way, mRNA expression would be altered due to insulin resistance as a result of obesity, rather than as a direct response to changes in adiposity. However, given the difficulties in identifying the presence of insulin resistance in cats [2], this hypothesis is difficult to prove.

These results also point towards nesfastin-1 in cats displaying similar characteristics to adiponectin, an adipokine whose expression decreases in obese and diabetic people and cats [49]. Similar to nesfatin-1, adiponectin is reported to increase insulin sensitivity and peripheral glucose uptake [47]. Further investigation into the potential interactions between nesfatin-1 and adiponectin expression in cats may be warranted.

There may be slightly decreased expression in these two tissues amongst the overweight and diabetic cats in comparison to the lean cats; however, these expression patterns are of questionable significance given the wide variance in expression seen in the lean cat group. The lack of a significant pattern in mRNA expression in skeletal muscle and abdominal adipose tissue may reflect the fact that these tissues are likely the target of nesfatin-1 activity. Nesfatin-1 enhances peripheral glucose uptake [61] and improves insulin sensitivity in peripheral tissues including skeletal muscle and adipose tissue [37]. Therefore, these tissues are receptive to the actions of nesfatin-1 but may not necessarily be important sites of nesfatin-1 production.

2.5.4 Strengths and Limitations

To our knowledge, this is the first study to evaluate and compare tissue nesfatin-1 mRNA expression in lean, overweight, and diabetic cats, and to consider how tissue nesfatin-1 expression may relate to differences in adiposity and glycemic control in cats. This provides new

insight into the role that nesfatin-1 plays in the pathophysiology of obesity and diabetes in cats. However, there were some limitations to this study. The main limitation is the sample size. The small sample size and the wide variance in tissue expression in all the groups makes it difficult to determine whether the changes seen between the lean, overweight, and diabetic cats are truly due to changes in glycemic control or adiposity, or due to other confounding factors influencing nesfatin-1 expression. For example, increases in plasma nesfatin-1 have been demonstrated in human that are hypertensive [55], and human patients with depression have shown increased levels of plasma nesfatin-1 [60], though it was not determined how this impacted underlying mRNA expression. Hypertension was not specifically evaluated in these cats; however, they did not show any evidence of cardiac disease, hyperthyroidism, severe renal disease, or other factors that cause hypertension in cats. The lean cats that were sampled were euthanized for apparent behavioural reasons, which potentially may have influenced nesfatin-1 expression. However, in laboratory animals, the induction of anxiety-like behaviours is only induced by ICV administration of nesfatin-1 [41], so there is no conclusive evidence to suggest it would impact gastrointestinal nesfatin-1 expression. Furthermore, we were not euthanizing cats specifically for the purposes of our study. Because of this, the only lean, otherwise healthy cats that were available for us to sample were cats that were euthanized for behavioural reasons, so this issue was considered unavoidable.

Finally, the nutritional status of all cats sampled was not known, and cats were not specifically fasted prior to euthanasia. 24 hour fasting has been shown to decrease NUCB2/nesfatin-1 mRNA expression in rat gastric endocrine cells [28], indicating that fasting or nutritional status has an impact on NUCB2/nesfatin-1 mRNA expression. Therefore, it is possible that any potential differences in the fasting status of the cats may have contributed to differences in mRNA expression, particularly in the stomach. It is possible that expression in the other tissues evaluated could also be affected by fasting status though this is considered less likely, particularly in regard to pancreatic expression, given that pancreatic nesfatin-1 may have different physiological functions to gastric nesfatin-1. Overall, larger sample sizes would allow us to evaluate the variability of expression within these individual groups with greater confidence, and therefore provide more support that differences between the groups are primarily due to differences in glycemic control and adiposity.

2.5.5 Future Studies

Future studies into nesfatin-1 expression would involve evaluation of additional gastrointestinal tissues to create a more holistic picture of NUCB2/nesfatin-1 expression. Analysis of mRNA expression in other gastrointestinal tissues, such as the liver or duodenum, may help uncover other sites where significant expression is present. Ideally, NUCB2/nesfatin-1 expression would be analysed in conjunction with formed nesfatin-1 protein expression, as well as plasma nesfatin-1 levels in each cat. Nesfatin-1 protein expression could be evaluated by immunoreactivity, which would help localize nesfatin-1 expression within the feline pancreas and other tissues. Histological examination of pancreatic tissue to evaluate the degree of islet cell pathology could also be pursued concurrently. Assessing mRNA and protein expression concurrently may be of particular benefit in cats given the previously identified discordance between NUCB2/nesfatin-1 mRNA expression and nesfatin-1 protein expression. Concurrent mRNA and protein expression evaluation would identify whether the changes in mRNA expression could result in changes in protein expression, and if this discordance between mRNA and protein expression in lean cats is also seen in overweight and diabetic cats. Concurrent measurement of plasma concentrations would assess whether the changes in tissue expression reflect changes that occur in peripheral nesfatin-1 concentrations. This would be of particular importance to further characterize the changes in pancreatic expression. If changes in pancreatic expression do not change in conjunction with changes in peripheral nesfatin-1, then it would suggest pancreatic expression does not significantly influence circulating nesfatin-1 and would instead hint at localized activity.

For examining peripheral tissues i.e., skeletal muscle and adipose tissue, which are the targets of nesfatin-1 and may not necessarily be major sites of nesfatin-1 production, it may be more valuable to assess changes in expression of the nesfatin-1 receptor, once it has been identified. More significant differences may be apparent in peripheral tissues of lean, overweight, and diabetic cats once expression of the receptor is evaluated.

2.5 Conclusion

In conclusion, the results of this study show there are differences in NUCB2/nesfatin-1 mRNA expression, particularly in gastric and pancreatic tissue and in diabetic cats. These patterns of expression could reflect changes in local pancreatic islet cell health and function in these cats. Diabetic cats appear to show a biphasic pattern of expression, with some diabetic cats maintaining NUCB2/nesfatin-1 expression similar to lean controls. Further investigation into changes in mRNA expression in feline gastrointestinal tissues, in conjunction with analysis of nesfatin-1 protein expression and circulating nesfatin-1 levels are needed to evaluate the significance of these changes in expression.

Transition

The previous chapter of this thesis evaluated tissue expression of NUCB2/nesfatin-1 mRNA expression in cats. We have also evaluated circulating concentrations of nesfatin-1 in lean, overweight, and diabetic cats, and this information is presented in the following chapter. The concurrent evaluation of circulating plasma nesfatin-1 concentrations allows us to demonstrate a more complete picture of nesfatin-1 expression in cats, and to further investigate the role of nesfatin-1 in the development of obesity and diabetes mellitus.

Chapter Three: Are Differences in Plasma Nesfatin-1 Concentrations in Cats Associated with Differences in Body Weight and Glycemic Control?

3.1 Abstract

Background: Obesity and diabetes mellitus are increasing issues in humans and domestic cats. Diabetes mellitus in cats shares similar clinicopathologic features to type 2 diabetes mellitus in humans. Nesfatin-1 is a recently discovered protein hormone that has been implicated in the development of obesity and diabetes, and alterations in plasma nesfatin-1 concentrations have been documented in type 2 diabetic humans. This study aimed to evaluate whether plasma nesfatin-1 concentrations in cats were associated with adiposity or insulin resistance, and to determine if nesfatin-1 concentrations were altered by diabetic treatment. Influences of sex and age on nesfatin-1 concentrations were also assessed.

Methods: Case control study. Plasma was collected from 13 lean and 13 obese non-diabetic cats, and eight newly diagnosed and five long term diabetic cats. Newly diagnosed diabetic cats were sampled before and after four weeks of diabetic treatment. Plasma nesfatin-1 levels were measured by a rat nesfatin-1 ELISA, which was validated for use with feline plasma.

Results: Median plasma nesfatin-1 concentrations in newly diagnosed and long-term treated diabetic cats showed a slight upward trend in comparison to lean and overweight non-diabetic cats, though the highest nesfatin-1 concentrations were seen in a subset of lean healthy cats. However, overall differences in plasma nesfatin-1 concentrations in lean, overweight, and diabetic cats were not statistically significant. Sex and age were also not significantly associated with plasma nesfatin-1 levels. Newly diagnosed diabetic cats showed a statistically significant decrease in nesfatin-1 concentrations following short-term treatment, which may suggest a link between decreased nesfatin-1 concentrations and diabetic treatment in cats.

Conclusions: Our results suggest that changes in plasma nesfatin-1 concentrations may be more related to diabetic status and the presence of insulin resistance as opposed to differences in adiposity or body condition, though further research is required to determine the significance of this relationship. There may be additional, currently unknown factors that increase nesfatin-1 concentrations in cats. Future studies may include longitudinal studies to investigate if nesfatin-1 concentrations change as cats develop increasing adiposity, insulin resistance, and eventually become clinically diabetic.

3.2 Introduction

The prevalence of obesity and obesity-related diabetes mellitus is increasing in both humans and cats [1]. Treatment of diabetic cats involves the administration of exogenous insulin and a high protein, low carbohydrate diet, as these diets are associated with reduced post prandial glucose and insulin concentrations [5]. However, management requires significant investments in time, money, and effort. This is not always feasible for owners and it is estimated around 30% of cats are euthanized within a year of diagnosis [4]. The ideal treatment outcome is the achievement of diabetic remission, defined as the maintenance of normoglycemia without the administration of exogenous insulin for at least four weeks [4]. However, remission rates are variable, ranging from 20 to 80% [6–8]. Another issue is that currently, no reliable marker exists to help identify cats that have a higher chance of remission, or to identify cats with insulin resistance that are not diabetic i.e., prediabetics [2,4]. If insulin resistance could be identified before diabetes occurs, treatment may be less laborious, avoiding the need for exogenous insulin or glucose monitoring. Similarly, if cat's that have a higher chance of achieving diabetic remission could be identified before starting treatment, this may provide added motivation to pursue aggressive treatment. Nesfatin-1 may have potential as such a marker.

Changes in circulating nesfatin-1 concentrations have been investigated in humans with obesity and diabetes mellitus. Plasma nesfatin-1 concentrations increase as body fat percentage increases in humans [46], and increases in plasma nesfatin-1 were also noted in people with newly diagnosed type 2 diabetes mellitus (T2DM) and those with impaired glucose tolerance i.e., prediabetics [52]. Nesfatin-1 has been shown to subsequently decrease in T2DM patients receiving treatment, suggesting a link between peripheral nesfatin-1 concentrations and both the development of T2DM and the resumption of adequate glycemic control [63]. IV injections of nesfatin-1 in mice have been shown to reduce blood glucose, but only in hyperglycemic states [45]. Furthermore, glucose, insulin, and nesfatin-1 levels were significantly higher in humans with impaired glucose tolerance, than healthy controls [54]. This suggests that increased nesfatin-1 concentrations may act as a compensatory mechanism, with plasma concentrations increasing in an attempt to maintain insulin function and glucose homeostasis when insulin sensitivity is impaired. The majority of cases of diabetes mellitus in cats share similar pathophysiological mechanisms to T2DM in humans i.e., insulin resistance and pancreatic ß-cell

dysfunction [2,4]. Thus, nesfatin-1 may play an important role in the development of obesity and diabetes mellitus in cats as well.

Despite the increasing prevalence of obesity and diabetes in cats, the role that nesfatin-1 plays in the pathogenesis of these conditions in cats is not well understood. A recent study has shown nesfatin-1 to be highly conserved amongst domestic species (greater than 90% similarity of the amino acid sequence in the bioactive core) and plasma nesfatin-1 concentrations in cats have been successfully measured using a commercially available ELISA [31]. Thus, the primary objectives of this study were the validation of a commercially available rat nesfatin-1 ELISA for use with feline plasma, and to investigate whether plasma nesfatin-1 concentrations differ between lean and overweight non-diabetic cats, and newly diagnosed diabetic cats. Secondary objectives included assessment of the effects of short-term and long-term diabetic therapy on plasma nesfatin-1 concentrations, and the comparison of plasma nesfatin-1 concentrations to other markers of adiposity and glycemic control including body weight, body condition score, serum glucose, serum fructosamine, and serum cholesterol. Given the associations between plasma nesfatin-1, adiposity and glycemic control in other species, we hypothesized that lean, overweight, and diabetic cats would have differing concentrations of plasma nesfatin-1. Nesfatin-1 concentrations were expected to be increased in overweight cats compared with lean cats, with higher concentrations in newly diagnosed diabetic cats compared to both lean and overweight non-diabetic cats. We also hypothesized that nesfatin-1 concentrations would decrease following short and long-term diabetic treatment.

3.3 Materials and Methods

3.3.1 Study Design, Case Selection and Control Groups

A case control study was performed. Forty-one cats were recruited through the Western College of Veterinary Medicine (WCVM) Veterinary Medical Centre. Animal use was approved by the University of Saskatchewan Animal Research Ethics Board (Animal Use Protocol Number 20190051). Inclusion criteria involved body condition score (BCS), age, amenability to restraint and venipuncture, and no prior history of behavioural disorders or cardiac disease. Exclusion criteria for all cats included cats that were not amenable to restraint without sedation, or those with evidence of pancreatic or systemic neoplasia.

Cats were placed into four separate study groups depending on their BCS or the presence of diabetes mellitus. Lean and overweight cats were recruited based on BCS; lean cats with a BCS of 4 or 5/9, and overweight cats with a BCS of 7 to 9/9 [67]. All condition scoring was performed by a single investigator (ES). Lean and overweight cats were confirmed as healthy by a clinical history, physical examination, and routine laboratory testing. Laboratory testing included a manual complete blood count, serum biochemistry, total thyroid hormone (tT4), serum fructosamine, and dipstick urinalysis. Exclusion criteria for lean and overweight cats based on laboratory findings included a moderate to marked anemia, inflammatory leukogram, moderate increases in ALP, ALT, and GGT, moderate hypercholesterolemia (>10 mmol/L), moderate hyperglycemia (>12 mmol/L), IRIS stage three or four renal disease, or hyperthyroidism. Thirteen lean cats were enrolled in the study. Fourteen overweight cats were initially enrolled, and one was subsequently excluded due to hyperthyroidism, so 13 overweight cats were included overall.

Diabetic cats were enrolled regardless of BCS. A clinical history, physical exam, screening blood work and urinalysis were also performed. A diagnosis of diabetes mellitus was based on clinical history and supportive laboratory findings including hyperglycemia (>12 mmol/L), glucosuria, and elevated fructosamine (>347 umol/L). Newly diagnosed diabetic cats were eligible for enrolment if they had not started insulin therapy or had been receiving insulin for less than one week. Long term diabetic cats were defined as being initially diagnosed with diabetes at least six months prior to enrolment. Exclusion criteria for new and long-term diabetic

cats included moderate to marked anemia, inflammatory leukogram, IRIS stage three or four renal disease, hyperthyroidism, and cats who developed diabetes as a result of recent glucocorticoid therapy. Nine newly diagnosed diabetic cats were initially enrolled, and one was excluded due to the discovery of pancreatic neoplasia, so eight newly diagnosed diabetic cats were included overall. Five long term diabetic cats were also enrolled in the study.

3.3.2 Blood and Urine Collection

Blood was collected once from lean, overweight, and long-term diabetic cats, while blood was collected twice from newly diagnosed diabetic cats. All blood was collected after cats were fasted for 8 to 12 hours and refrigerated at 4°C within 10 minutes of collection. Plasma samples for nesfatin-1 analysis were collected in EDTA tubes (Ref 367841; Becton Dickinson and Company, NJ) and serum samples for biochemistry, tT4, and fructosamine were collected into serum separator tubes (Ref 367983; BD, NJ). Urine was collected via ultrasound guided cystocentesis. For all diabetic cats, blood glucose and ketone levels were analyzed by a handheld glucometer (Alpha Trak 2, Abbott, CA) and ketometer (Freestyle Optimum Neo, Abbott, CA) immediately following collection. PCV and TP were measured along with a Kwik Diff™ (catalogue number 9990701; Thermo Scientific, WA) stained blood smear for manual slide evaluation. Plasma and serum were centrifuged within 30 minutes of collection and stored at -80°C until required. Urine was analysed within 30 minutes or stored at 4°C and warmed to room temperature before analysis. Urine specific gravity was measured by refractometry, and chemical constituents were measured by dipstick (Chemstrip 9, Roche, Quebec). If abnormalities were found on the dipstick e.g., hematuria or proteinuria, the urine sediment was assessed cytologically.

Newly diagnosed diabetic cats at their first appointment were prescribed exogenous insulin administration and dietary modifications. Seven cats were started on Lantus Toujeo® insulin pens 2 IU BID, and one cat was prescribed Glargine® insulin as it had already been started four days prior to enrolment. Seven diabetic cats were switched to Purina® Diabetic Management food after a five-day transition from their original diet. One cat was placed on Purina® NF Renal Function food due to concurrent IRIS stage two renal disease.

The second appointment for the newly diagnosed diabetic cats occurred four to five weeks after the first appointment. Blood was collected into EDTA and serum separator tubes, then processed and stored as previously described. Serum was submitted for repeat cholesterol and fructosamine measurement. A subcutaneous glucose monitoring device (Freestyle Libre, Abbott, CA) was fitted, and interstitial glucose readings were recorded digitally for up to 14 days. Interstitial glucose was recorded as an average of the first three days of measurement, so that glucose levels were representative of glycemic control closer to the time of the second blood collection.

3.3.3 Validation of the Nesfatin-1 ELISA

Assay validation was performed using plasma samples from lean and overweight cats collected as part of an unrelated study involving collaborators at the University of Calgary Faculty of Veterinary Medicine (UCVM) and the WCVM. All samples were stored at -80^{o}C. Validation was performed on a rat nesfatin-1 ELISA kit (catalogue number EK-003-22; Phoenix Pharmaceuticals Inc, CA) run in accordance with manufacturer's instructions. As purified feline nesfatin-1 is not commercially available, feline plasma samples were run individually to determine their concentrations, and then pooled to create low, medium, and high concentration samples.

Assay precision was determined using the pooled samples. Intra-assay coefficient of variation (CoV) was calculated by running the pooled samples five times in duplicate. Inter-assay CoV was assessed by running pooled samples five times on five different days in duplicate. Targeted intra and inter-assay CoV was less than 10%. Assay accuracy was measured by sample parallelism, linearity under dilution, and a modified spiked recovery test. Parallelism was assessed using three individual feline plasma samples. Sample concentration was measured in duplicate, then serially diluted (3:4, 1:2, 1:4, 1:8) with expected values compared to the measured values. For the spiked recovery test, two individual feline plasma samples were used as purified feline nesfatin-1 is not commercially available. The samples were mixed in three ratios (1:5, 1:1, and 4:5) and analysed in duplicate, and expected results were compared to the measured values. Limit of detection was calculated from five replicated zero standards (assay

buffer) as a mean plus three standard deviations. For test parallelism, a sample recovery of 70 to 130% was targeted for the measured dilutions in comparison to the expected values. An r^2 value of greater than 0.95 was targeted for linearity under dilution. For the modified spiked recovery test, we targeted a sample recovery of 80 to 120%.

3.3.4 Feline Plasma Nesfatin-1 Measurement

All plasma samples were run with the previously mentioned rat nesfatin-1 ELISA (catalogue number EK-003-22; Phoenix Pharmaceuticals Inc, CA) according to manufacturers' instructions. Feline plasma was thawed and centrifuged at 10,000 x *g* for 10 minutes to precipitate any visible lipemia present [68]. Thirty microlitres of each sample was taken and combined with 90 uL of the assay buffer in a separate Eppendorf tube for a 1:4 dilution.

3.3.5 Statistical Analysis

Descriptive statistics for nesfatin-1 levels in the lean, overweight, and diabetic cats were evaluated. Normal distribution of plasma nesfatin-1 levels was assessed by a Shapiro-Wilk test. Plasma nesfatin-1 concentrations were compared between the original study groups, lean cats, overweight cats, and newly diagnosed diabetic cats at their initial presentation. The effects of body condition and diabetes were then assessed individually. BCS was transformed into a categorical variable, with cats classified as either being lean (BCS less than 6/9), or overweight (BCS greater than or equal to 6/9), regardless of diabetic status. All overweight non-diabetic cats had a BCS greater than or equal to 7/9, a single diabetic cat had a BCS of 6/9. For diabetic status, cats were classified as either diabetic or non-diabetic, regardless of their body condition. Cats were classified as young (less than six years of age), middle age (6 to 9 years of age), or old (greater than nine years of age). Differences in plasma nesfatin-1 according to group, body condition, diabetic status, age, and sex were assessed by a Kruskal-Wallis ANOVA. Associations between plasma nesfatin-1 and fructosamine, glucose, body weight, and cholesterol were evaluated with linear regression. Further interactions between individual continuous and categorical variables were assessed by multivariate regression analysis. Variables were included

in the multiple regression model if their P-value was less than 0.3, and the significance of these different models was assessed in comparison to a null model by likelihood ratio. Correlations between these variables were assessed by the variance inflation factor (VIF).

Statistical analysis was performed without the four samples that had the highest plasma nesfatin-1 concentrations, as these samples were considered potential outliers. This data was assessed similarly as above, though without these four values the data was normally distributed. Plasma nesfatin-1 levels were compared between lean, overweight and diabetic cats by a one-way ANOVA with Bonferroni correction. Differences in nesfatin-1 according to body condition, age, diabetic status, and sex were compared with a t-test. Associations between plasma nesfatin-1 and fructosamine, glucose, body weight, and cholesterol were assessed by linear regression, and multivariate regression analysis was performed.

Descriptive statistics for analysis of nesfatin-1 concentrations in diabetic cats before and after four weeks of treatment were performed and normality was assessed with a Shapiro-Wilk test. One cat had a fructosamine concentration far lower than what was expected clinically, and this fructosamine values was not included in the statistical analysis as analytical or laboratory error could not be excluded. Changes in nesfatin-1, body weight, glucose, and fructosamine were assessed with a paired t-test, and changes in cholesterol were assessed by a Wilcoxon Signed-Rank Test. Associations between changes in nesfatin-1 concentrations and changes in body weight, fructosamine, glucose, and cholesterol were assessed by linear regression. Multivariate regression was performed as to assess if there were significant interactions between variables. Statistical significance for all tests was set at $P < 0.05$

3.4 Results

3.4.1 Nesfatin-1 ELISA Validation Results

The nesfatin-1 ELISA kit used in this study showed acceptable validation characteristics when using feline plasma, though dilution of the samples created additional variation. The undiluted feline nesfatin-1 plasma concentrations were frequently higher than the listed linear range of the assay (linear range 0.92 – 12.7 ng/mL). Despite this, the intra and inter-assay CoVs were all well within the targeted CoV of less than 10% in the low, medium, and high concentration samples (Table 2.1). The modified spiked recovery test showed an acceptable average sample recovery of 106% (Table 2.1). Serial dilution of three unknown samples with the assay buffer displayed linearity under dilution (slope $r^2 > 0.95$). However, these serial dilutions showed increasing sample recovery percentage in comparison to the expected nesfatin-1 concentration (Table 2.2), i.e., relative sample recovery increases as a dilution factor increases. This increasing recovery is consistent with a negative matrix effect associated with the feline plasma, and at higher dilutions (1:2 or greater) the variation was higher than the target parallelism of 70 to 130%. As this matrix effect showed different degrees of strength at different dilutions, all subsequent plasma samples were run with the same dilution factor (1:4). This dilution factor was chosen to ensure measured nesfatin-1 concentrations fell within the linear range of the assay. The variable matrix effect associated with dilution may limit use of the assay in a diagnostic setting but was considered acceptable for the purposes of this study.

	Intra-Assay Variation			Inter-Assay Variation		
	Low	**Medium**	**High**	**Low**	**Medium**	**High**
Mean concentration (ng/mL)	10.6	17.86	23.87	12.83	21.09	27.19
Coefficient of Variation (%)	3.62	4.42	3.77	6.45	1.7	2.02
Limit of Detection (ng/mL)	0.32					
Modified Spiked Recovery Test Average Sample Recovery	106%					

Table 3.1: Intra-assay and inter-assay variation, limit of detection, and modified spiked recovery test for feline nesfatin-1 samples using a rat nesfatin-1 ELISA. Intra and Inter-assay CoVs were determined with pooled feline plasma samples of Low, Medium, and High nesfatin-1 concentrations

Sample Dilution	Sample 1 Recovery %	Sample 2 Recovery %	Sample 3 Recovery %	Mean %
1:1	-	-	-	
3:4	116	121	120	119
1:2	135	144	135	138
1:4	182	181	214	192
1:8	194	224	281	233
Slope r^2	0.97	0.96	0.97	

Table 3.2: Linearity under dilution (r^2) and parallelism of nesfatin-1 concentrations (Measured concentration divided by the expected or calculated nesfatin-1 concentration for each dilution) for three separate feline plasma samples

3.4.2 Plasma Nesfatin-1 Levels in Lean, Overweight and Diabetic Cats

The most striking feature seen in the initial data between the defined groups was the increased variance in the lean cat group (Figure 3.1 and Table 3.3) (variance = 7.4), which was higher than any other group (variance ranged from 0.5 – 0.7). The observed median plasma nesfatin-1 concentrations were lowest in the lean and overweight cat groups. However, the cats with the four highest individual plasma nesfatin-1 concentrations were all in the lean cat group. Observed median nesfatin-1 concentrations in newly diagnosed (5.8 ng/mL) diabetic cats were slightly higher than both lean and overweight non-diabetic cats, though this difference was not statistically significant (P = 0.21). Median plasma nesfatin-1 concentrations in long-term diabetic cats (6.4 ng/mL) were also higher than both lean and overweight, non-diabetic cats, though this difference was also not statistically significant (P = 0.16).

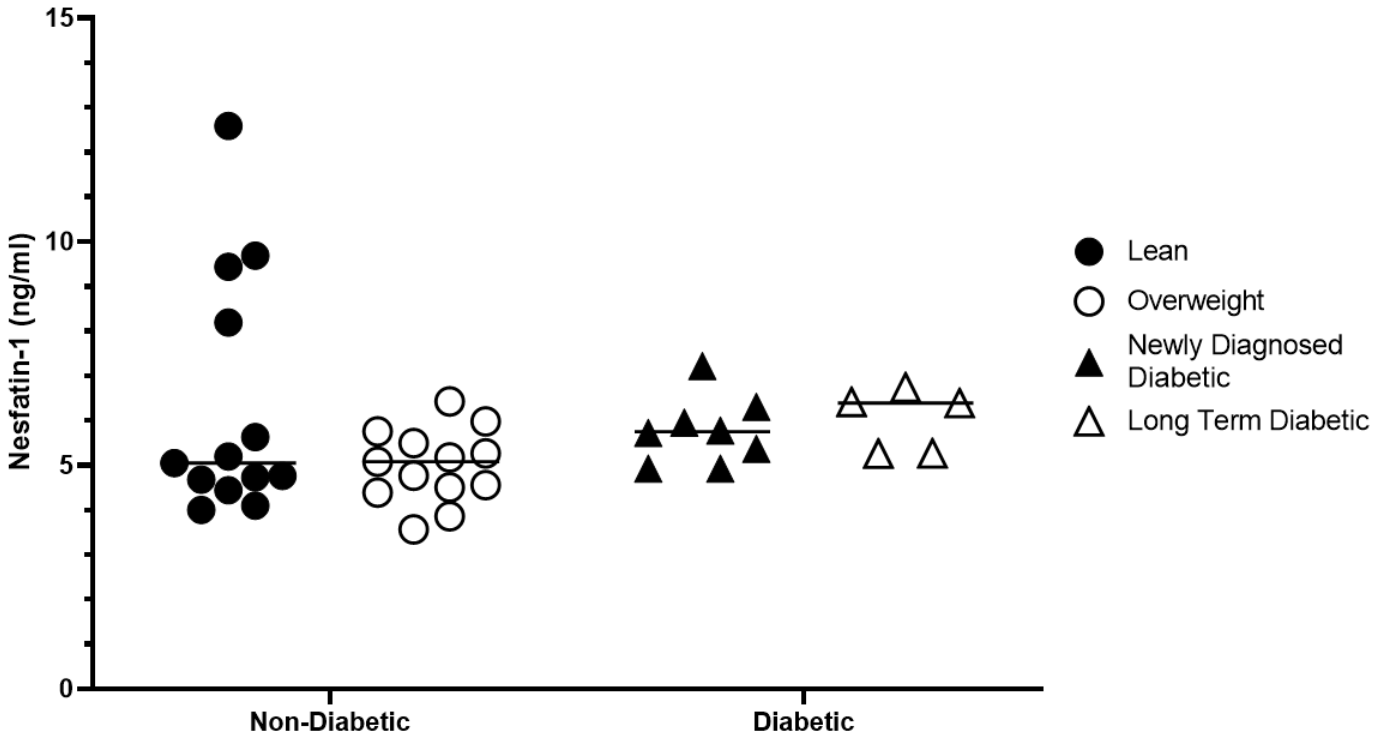


Figure 3.1: Plasma nesfatin-1 concentrations in lean (n = 13) and overweight (n = 13) non-diabetic cats from an owned cat population, and newly diagnosed (n = 8) and long-term (n = 5) diabetic cats referred to the Western College of Veterinary Medicine Veterinary Medical Centre between August 2019 and March 2020.

3.4.3 Relationship Between Plasma Nesfatin-1 and Other Patient Variables

Plasma nesfatin-1 levels in all cats were assessed in relation to characteristics associated with adiposity and glycemic control. Overall, no individual variable showed a statistically significant relationship to plasma nesfatin-1 concentrations (Table 3.3). The closest relation seen was that of plasma nesfatin-1 concentrations and diabetic status (P = 0.11), followed by group (P = 0.21) and body condition (P = 0.33). A poor relationship was seen with plasma nesfatin-1 and body weight (coefficient -0.3, P = 0.1, linear regression). This model also had low r^2 value (0.09) and lacked normally distributed residuals. Nesfatin-1 was not found to be associated with serum cholesterol (coefficient 0.01, P = 0.5, linear regression), serum fructosamine (coefficient -0.01, P = 0.96, linear regression) and serum glucose (coefficient -0.01, P = 0.98, linear regression). Multivariate regression analysis was attempted including the body weight and diabetic status variables. The 'Group' variable was not included in multi-variate analysis as this variable combines the effects of changes in body weight/ body condition and diabetic status. Using multivariate regression, a model with significant independent risk factors could not be created and no significant associations between variables and nesfatin-1 concentrations were seen. No model had acceptable fit and normally distributed residuals (data not shown).

Characteristics of cats in the study	Characteristic Sub-Category	Median Plasma Nesfatin-1 (Min-Max) (ng/mL)	Variance	P-Value
Group	**Lean (n = 13)**	5.1 (4 – 12.6)	7.4	0.21
	Overweight (n = 13)	5.1 (3.6 – 6.4)	0.7	
	Newly Diagnosed Diabetic (n = 8)	5.8 (4.9 – 7.2)	0.6	
	Long Term Diabetic (n = 5)*	6.4 (5.3 – 6.7)	0.5	0.16
Diabetic Status	**Non-Diabetic (n = 26)**	5.1 (3.6 – 12.6)	4.4	0.11
	Newly Diagnosed Diabetic (n = 8)	5.8 (4.9 – 7.2)	0.6	
Body Condition (1-9 Scale)	**Less Than 6/9 (n = 18)**	5.3 (4 – 12.6)	5.4	0.33
	Greater Than or Equal To 6/9 (n = 16)	5.1 (3.6 – 6.4)	0.6	
Age	**Less than 6 years of Age (n = 11)**	4.7 (3.9 – 9.7)	2.6	0.4
	6-9 Years of Age (n = 14)	5.2 (4.1 – 12.6)	5.3	
	Greater than 9 Years of Age (n = 9)	5.4 (3.6 – 8.2)	1.9	
Sex	**Male (n = 20)**	5.4 (3.6 – 8.2)	1.4	0.38
	Female (n = 14)	5.0 (3.9 – 12.6)	4.8	

Table 3.3: Associations between plasma nesfatin-1 concentrations and selected characteristics of lean and overweight non-diabetic cats, and newly diagnosed diabetic cats from an owned cat population referred to the Western College of Veterinary Medicine Veterinary Medical Centre between June 2019 and March 2020, Kruskal-Wallis ANOVA.

*The long-term diabetic cats (n = 5) were not included in the ANOVA analysis and are presented in this table for descriptive purposes only

3.4.4 Plasma Nesfatin-1 Data Without Potential Outliers

To understand the influence of the four extreme values of nesfatin-1 in the lean group on the overall analysis a subset analysis was run excluding them (Table 3.4). These four lean cats had plasma nesfatin-1 concentrations of 8.2, 9.4, 9.7, and 12.6 ng/mL respectively. In this subset analysis there was a statistically significant association found between nesfatin-1 concentrations and diabetic status (P = 0.01), and between nesfatin-1 concentrations in the lean cat and newly diagnosed diabetic cat group (P = 0.02). An association was also found between plasma nesfatin-1 concentrations and both fructosamine (Co-efficient 0.3, P = 0.01, linear regression) and glucose (Co-efficient 0.3, P = 0.03, linear regression). There were poor associations seen between nesfatin-1 and cholesterol (Co-efficient 0.06, P = 0.28, linear regression), and nesfatin-1 and body weight (Co-efficient -0.07, P = 0.44, linear regression). Multivariate regression was attempted including diabetic status and serum cholesterol as variables. Fructosamine and glucose were not included, as the variable 'diabetic status' is defined by changes in glucose and fructosamine. Therefore, these variables are considered colinear, with diabetic status being an intermediary variable. This multivariate model was not significant according to likelihood ratio, though cholesterol was found to be a confounding variable for diabetic status. Diabetic status remained an independent risk factor in this multivariate model (Co-efficient 1.2, P = 0.01).

Variable	Variable Sub-Category	Median Plasma Nesfatin-1 (Min-Max) (ng/mL)	Variance	P-Value
Group	**Lean (n = 9)**	4.7 (4 – 5.6)	0.3	0.02
	Overweight (n = 13)	5.1 (3.6 – 6.4)	0.7	
	Newly Diagnosed Diabetics (n = 8)	5.8 (4.9 – 7.2)	0.6	
	Bonferroni Correction Values		**Lean**	**Overweight**
	Newly Diagnosed Diabetic		0.02	0.07
	Lean			1
Diabetic Status	**Non-Diabetic (n = 22)**	4.8 (3.6 – 6.4)	0.5	0.01
	Diabetic (n = 8)	5.8 (4.9 – 7.2)	0.6	
Body Condition (1-9 Scale)	**Less Than 6/9 (n = 14)**	5.0 (4 – 7.2)	0.8	0.85
	Greater Than or Equal To 6/9 (n = 16)	5.1 (3.6 – 6.4)	0.6	
Age	**Less than 5 years of Age (n = 10)**	4.6 (3.9 – 6.0)	0.6	0.35
	6-9 Years of Age (n = 12)	5.1 (4.1 – 6.4)	0.4	
	Greater than 9 Years of Age (n = 8)	5.2 (3.6 – 7.2)	1.2	
Sex	**Male (n = 17)**	5.2 (3.9 – 6.4)	0.6	0.76
	Female (n = 13)	5.0 (3.6 – 7.2)	0.8	

Table 3.4: Correlations between plasma nesfatin-1 concentrations and selected characteristics of lean and overweight non-diabetic cats from an owned cat population, and newly diagnosed diabetic cats referred to the Western College of Veterinary Medicine Veterinary Medical Centre between August 2019 and March 2020, One-Way ANOVA with Bonferroni correction (Group), and t-test (Diabetic status, body condition, age, sex). The four highest individual plasma nesfatin-1 concentrations have been omitted, and the long-term diabetic cats (n = 5) were not included in the statistical analysis.

3.4.5 Changes in Plasma Nesfatin-1 Concentrations and Other Variables in Diabetic Cats at Initial Presentation and After Four Weeks of Diabetic Treatment

Nesfatin-1 levels in diabetic cats before and after treatment showed similar, small levels of variance between the pre- and post-treatment nesfatin-1 concentrations. Median plasma nesfatin-1 concentrations before treatment were higher than concentrations following treatment (5.8 ng/mL pre-treatment, 4.7 ng/mL post-treatment, Figure 3.2 and Table 3.5) and all eight individual cats had higher nesfatin-1 levels before treatment. This decrease in nesfatin-1 concentrations following treatment was statistically significant ($P < 0.01$) (Table 3). A statistically significantly increase in body weight ($P < 0.01$) was seen, along with statistically significant decreases in glucose ($P < 0.01$), fructosamine ($P < 0.01$), and cholesterol ($P = 0.01$) concentrations following treatment.

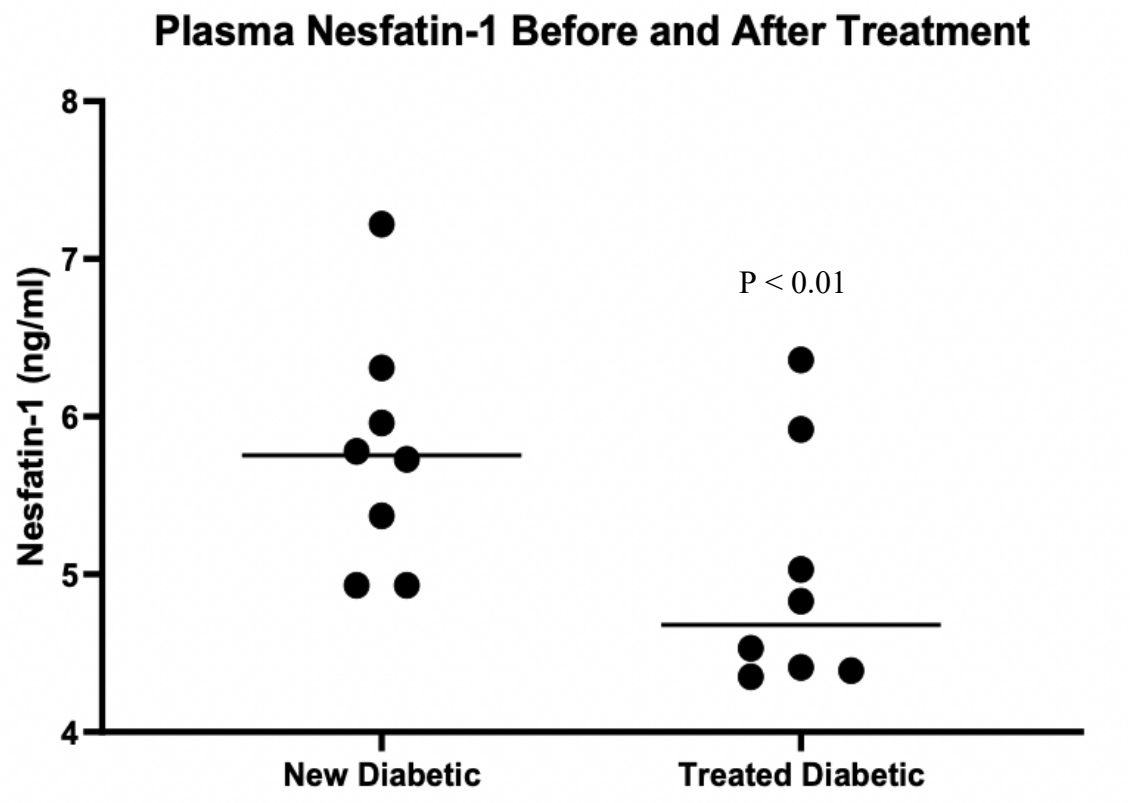


Figure 3.2: Plasma nesfatin-1 concentrations of newly diagnosed diabetic cats (n = 8) presenting to the WCVM between August 2019 and March 2020, before treatment and after four weeks of diabetic treatment ($P < 0.01$), Paired t-test

Group	Median Plasma Nesfatin-1 (Min – Max) (ng/mL)			Variance
Diabetic Pre-Treatment	5.8 (4.9 – 7.2)			0.6
Diabetic Post-Treatment	4.7 (4.3 – 6.4)			0.8
Difference Pre & Post Tx	**Mean Diff**	**Std Dev**	**95% CI**	**P-Value**
ΔNesfatin-1 (ng/mL)	-0.8	0.5	-1.2 - -0.4	<0.01
ΔWeight (kg)	0.4	0.3	0.2 – 0.6	<0.01
ΔGlucose (mmol/L)	-14	7.5	-20 - -7.3	<0.01
ΔFructosamine (umol/L)	-170	80	-238 - -105	<0.01
ΔCholesterol (mmol/L)	-1.7	-	-	0.01

Table 3.5: Plasma nesfatin-1 concentrations in newly diagnosed client owned diabetic cats (n = 8) presenting to the WCVM between August 2019 and March 2020, before and after four weeks of diabetic treatment, and differences in selected variables in these cats following diabetic treatment, Paired t-test (nesfatin-1, Weight, Glucose, Fructosamine) and Wilcoxon Signed-Rank test (Cholesterol)

3.4.6 Correlations Between Changes in Plasma Nesfatin-1 and Changes in Other Variables

Changes in plasma nesfatin-1 concentrations before and after treatment were compared to changes in body weight, fructosamine, glucose, and cholesterol (Table 3.6). The closest correlation was seen with changes in glucose (coefficient -0.45, P = 0.09, linear regression), with slight associations seen with changes in fructosamine (coefficient -0.39, P = 0.26, linear regression) and changes in body weight (coefficient -1, P = 0.19, linear regression). No correlation was apparent with cholesterol (coefficient 0.003, P = 0.98, linear regression). Multivariable regression analysis was performed, including glucose and fructosamine. Changes in fructosamine were highly correlated with changes in body weight (VIF = 12). Improved glycemic control (reflected by fructosamine) was considered to be an independent variable,

whereas the increase in body weight is dependent on improved glycemic control, therefore fructosamine was included in the model at the expense of body weight. No multivariate models were significant and no significant independent risk factors were identified (data not shown).

Variables	Coefficient	95% CI	P-Value (r^2)
ΔNesfatin-1 with ΔWeight	-1.0	-2.7 – 0.66	0.19 (0.27)
ΔNesfatin-1 with ΔFructosamine	-0.39	-1.2 – 0.39	0.26 (0.25)
ΔNesfatin-1 with ΔGlucose	-0.45	-0.99 – 0.09	0.09 (0.41)
ΔNesfatin-1 with ΔCholesterol	0.003	-0.39 – 0.40	0.98 (<0.01)

Table 3.6: Correlations between the changes in plasma nesfatin-1 concentrations and changes in selected variables in newly diagnosed diabetic cats at their initial presentation and following four weeks of treatment, linear regression.

3.5 Discussion

This study evaluated plasma nesfatin-1 concentrations in lean, overweight, and diabetic cats, and a rat nesfatin-1 ELISA was validated for use with feline plasma. The results of this study demonstrated a statistically significant decrease in plasma nesfatin-1 concentrations following treatment of newly diagnosed diabetic cats. No statistically significant differences in plasma nesfatin-1 concentrations were seen between lean and obese non-diabetic cats, and newly diagnosed diabetic cats, though overall plasma nesfatin-1 concentrations appeared marginally increased in diabetic cats in comparison to non-diabetic cats. However, the four highest individual plasma nesfatin-1 concentrations occurred in lean cats.

3.5.1 Plasma Nesfatin-1 Concentrations in Lean Cats

We found greater variance in plasma nesfatin-1 concentrations in lean cats compared to the other groups. While the median nesfatin-1 concentrations in the lean and overweight groups were below those of the newly diagnosed and long-term diabetics, the four highest nesfatin-1 concentrations in individual cats were all in the lean cat group. A laboratory error for these values was considered highly unlikely as there were no major discrepancies between the duplicate samples. The high concentration samples were also positioned in three separate columns, so an error in plate washing was considered unlikely. The variance in nesfatin-1 concentrations in this lean cat group is generally higher than the variance seen in nesfatin-1 concentrations from control groups in human diabetic studies [52,53,62], though a direct comparison of variance is difficult given that plasma nefatin-1 concentrations in cats appear to be much higher than in humans.

Interestingly, two of the four highest concentrations came from cats that lived in the same household, which may suggest an environmental link. However, one cat with a high nesfatin-1 concentration lived with a cat whose nesfatin-1 levels were closer to the median concentration. Follow up was conducted into all cats that were enrolled in our study approximately 18 months after sampling, and no associations (behavioural, medical, or dietary) could be found between these cats with high nesfatin-1 concentrations. Three of the cats had been clinically well in the year since sampling, while one cat was diagnosed with pododermatitis one month after sampling,

and also developed a lower urinary tract obstruction six months after sampling. The underlying cause of these high nesfatin-1 concentrations is not apparent, though it may suggest there are reasons other than body condition and alterations in glycemic control that can unpredictably influence plasma nesfatin-1 concentrations. Interestingly, a previous study documented a population of lean cats that had low insulin sensitivity, and were therefore at increased risk of developing obesity and impaired glucose tolerance [69]. Speculatively, if nesfatin-1 concentrations are related to differences in glycemic control, then these cats with increased nesfatin-1 concentrations could reflect a population of lean cats with comparatively decreased insulin sensitivity. However, if this were the case, it would be expected that similarly high nesfatin-1 concentrations should be seen in the individual diabetic cats i.e., cats that should have significantly decreased insulin sensitivity. It is also possible that these high nesfatin-1 concentrations could also be seen in overweight and diabetic cats, but given the small sample size of our study were not present in the subset of overweight and diabetic cats.

3.5.2 Plasma Nesfatin-1 Concentrations in Overweight Cats

No significant differences in nesfatin-1 concentrations were identified in overweight cats in comparison to lean cats. It was expected that nesfatin-1 concentrations would be increased in overweight or obese non-diabetic cats given that obese humans have increased nesfatin-1 levels compared with healthy controls [46]. However, the overweight cats in our study had similar nesfatin-1 concentrations to the lean cats, suggesting that obesity may not have as much of an effect on plasma nesfatin-1 in cats. There are several potential reasons for this. The differences between lean and overweight cats may be small, in which case a larger sample size would be required to demonstrate a difference. Additionally, it is known that cats compensate for the insulin resistance for long periods of time, and the presence of insulin resistance in cats is difficult to identify [2]. No reliable marker of insulin resistance is currently available in cats, as methods used in humans e.g., intravenous glucose tolerance testing or endogenous insulin concentrations, are unreliable for use in cats [2]. Therefore, it is possible that the overweight cats enrolled in our study did not have significant insulin resistance or were already compensating adequately for any insulin resistance present, in which case nesfatin-1 concentrations may not be altered. On follow up 18 months after sampling, no overweight cats had developed diabetes

mellitus. Future long-term longitudinal studies following lean and overweight or obese cats would be helpful to determine if there are significant differences or changes in nesfatin-1 concentrations between cats that develop diabetes and those that do not. If so, nesfatin-1 may be useful as a proxy for identifying insulin resistance as it develops, and potentially before the development of clinical diabetes.

3.5.3 Plasma Nesfatin-1 Concentrations in Diabetic and Non-Diabetic Cats

Although the differences in plasma nesfatin-1 concentrations between the lean, overweight, and diabetic cat groups were not statistically significant, the greatest difference in nesfatin-1 concentrations was found to be between diabetic and non-diabetic cats, independent of body condition. Median nesfatin-1 concentrations in newly diagnosed and long-term diabetic cats were both higher than the lean and overweight non-diabetic cats. However, the four lean non-diabetic cats with high nesfatin-1 concentrations created a skewed data set and impacted this association between diabetic and non-diabetic cats. Without these four values, a statistically significant difference between newly diagnosed diabetic and non-diabetic cats could be seen. Similarly, without these four cats, a significant relationship between plasma nesfatin-1 concentrations and glucose ($P = 0.98$ with these outliers, $P = 0.03$ without) and fructosamine ($P = 0.96$ with outliers, $P = 0.01$ without) was seen. This large discrepancy in significance suggests that there are unmeasured confounding or interacting factors in these four lean cats that influence nesfatin-1 concentrations. While the significance of the data without these four cats is questionable, it could suggest a potential association between the development of diabetes and increased plasma nesfatin-1 concentrations in cats. Similar results have been reported in newly diagnosed type 2 diabetic humans [63], and suggest that increased nesatin-1 concentrations are a compensatory mechanism to help maintain insulin sensitivity and peripheral glucose uptake. Additionally, IV nesfatin-1 injections were found to reduce blood glucose levels in hyperglycemic mice [45], further supporting its antihyperglycemic effects. However, given the limited sample size and overlap between the study groups, the results of this study should be interpreted with caution. Further investigation, in particular a larger sample of diabetic cats is required to confirm the positive correlation between diabetes and plasma nesfatin-1 concentrations in cats.

3.5.4 Relationship Between Changes in Nesfatin-1 and Changes in Body weight and Glycemic Control Following Treatment in Newly Diagnosed Diabetic Cats

Newly diagnosed diabetic cats showed a statistically significant decrease in plasma nesfatin-1 concentrations following four-weeks of diabetic treatment. This pattern reflects the patterns seen in human diabetics, who have decreased nesfatin-1 concentrations following diabetic treatment in comparison to newly diagnosed diabetic patients [63]. This could again reflect nesfatin-1 compensating for the insulin resistance present in newly diagnosed diabetics, as previously mentioned. As glycemic control, and possibly the severity of ß-cell glucose toxicity, improves with treatment, this compensatory mechanism abates, and plasma nesfatin-1 concentrations decrease.

The underlying reason for the decrease in nesfatin-1 concentrations following treatment in the newly diagnosed diabetic cats is uncertain as there were no statistically significant correlations between changes in nesfatin-1 concentrations and other variables. However, the changes appeared to be more related to changes in glycemic control as opposed to changes in body weight. Interestingly, changes in nesfatin-1 are more closely related to changes in blood glucose than to changes in fructosamine. This discrepancy may reflect individual variation and the small sample size; however, it may also be secondary to the differences in the time scale of the two tests. Fructosamine measures glycated protein as an indicator of average blood glucose levels over the previous three weeks [70], whereas blood and interstitial glucose reflects the current glucose levels around the time of sample collection. The effect of stress hyperglycemia on blood glucose concentrations was minimized by the use of the Freestyle Libre® sub-cutaneous glucose monitoring device and collection of blood glucose data at home in a non-stressful environment. Furthermore, the half-life of plasma nesfatin-1 in mice is approximately 10 minutes [40], and this short half-life suggests plasma nesfatin-1 may align better with measurements of short-term glycemic control such as blood glucose.

Nesfatin-1 levels were not found to be closely associated with body weight or body condition in either diabetic or non-diabetic cats in our study. Conversely, nesfatin-1 concentrations are reported to increase as body mass index (BMI) increases in humans [46]. Interestingly, the one cat that did not gain weight after four weeks of diabetic treatment also had the smallest decrease in plasma nesfatin-1. Altogether, these findings suggest that nesfatin-1 may

operate independently of body weight and adiposity in cats, and instead more closely reflect glycemic control in this species. Further research to confirm this is required.

Dietary changes, as instituted in the newly diagnosed diabetic cats, may also be a confounding factor. In dogs, previous research has shown that high fibre/ high protein diets increased gastric expression of NUCB2/nesfatin-1, although concurrent plasma nesfatin-1 concentrations were not significantly altered [30]. Given that similar dietary changes designed to improve glycemic control, namely increased protein content, are prescribed to diabetic cats, it is difficult to determine the exact impact of dietary changes alone on plasma nesfatin-1 concentrations, if any. If diet alone has a significant impact on plasma nesfatin-1 concentrations and overall glycemic control, this could have implications for overweight cats and those at risk of developing diabetes, similar to human pre-diabetic patients where dietary modifications are frequently instituted as a first line treatment [12].

3.5.5 Plasma Nesfatin-1 Concentrations in Newly Diagnosed and Long-Term Diabetic Cats

Patterns in plasma nesfatin-1 concentrations in newly diagnosed and long-term diabetic cats differed from what was expected. In a recent meta-analysis, circulating nesfatin-1 levels were elevated in newly diagnosed type 2 human diabetic patients but decreased in diabetic patients receiving anti-diabetic treatment, with the duration of anti-diabetic treatment thought to partially contribute to decreased nesfatin-1 concentrations [63]. This pattern was not seen in our study, as both newly diagnosed and long-term diabetic cats had similar, increased, plasma nesfatin-1 concentrations. The small sample size may have impacted our results though it is possible that physiological differences in cats play a role. For example, even cats who respond well to treatment and achieve diabetic remission have abnormal glucose metabolism [9,71]. Furthermore, healthy cats show blunted insulin responses to high glucose loads in comparison to dogs. This is thought to be related to a lack of glucokinase activity, a metabolic adaptation that is also found in other obligate carnivores [72]. Speculatively, these metabolic differences could reflect a continued need for increased nesfatin-1 secretion as a result of differences in insulin

secretion and function in healthy and diabetic cats, which could also suggest that cats are able to maintain increased nesfatin-1 secretory ability in the long term.

Another possibility is that the diabetic treatment administered in the long-term diabetic cats may be less effective in comparison to human diabetic patients receiving treatment. This may be reflected by comparatively poorer glycemic control and therefore higher nesfatin-1 concentrations. However, the aforementioned meta-analysis only demonstrated a relationship between nesfatin-1 concentrations and whether the patients had received treatment or not, an association was not found between nesfatin-1 concentrations and insulin resistance in human T2DM [63]. This suggests that whether the patient is receiving treatment is a greater determinant of nesfatin-1 concentrations than the efficacy of the treatment. Similarly, the long-term diabetic cats in our study had varying degrees of glycemic control (based on varying serum fructosamine concentrations) but similar nesfatin-1 concentrations.

A major issue limiting interpretation is that we do not know the nesfatin-1 concentrations of the long-term diabetic cats when they were initially diagnosed. It is possible that all the long-term diabetic cats sampled may have had even higher nesfatin-1 concentrations at diagnosis. If this were the case, then these values could still be consistent with what is seen in people i.e., nesfatin-1 concentrations are decreased with diabetic treatment. Further investigation is required to assess any differences that may exist between newly diagnosed and long-term diabetics, and the relationship between plasma nesfatin-1 and glycemic control in these patients.

3.5.6 Strengths and Limitations

To our knowledge, this is the first study to evaluate changes in plasma nesfatin-1 concentrations in cats, particularly in relation to body condition and diabetes mellitus, and how nesfatin-1 concentrations change with diabetic treatment. The information in this study also provides a basis to further investigate the links between nesfatin-1, adiposity, and glycemic control and insulin resistance. However, there were several limitations. The small sample size of this study, particularly in the newly diagnosed diabetic cat group, made it difficult to determine statistically significant differences between the groups, if they do in fact exist. The sample size in this diabetic cat group could have been expanded to include the long-term treated diabetic cats.

However, given the differences in nesfatin-1 levels before and after treatment in humans [63], and with the changes in nesfatin-1 that occurred after treatment in our cats, it was considered that combining these groups would add additional variability in glycemic control and pancreatic function that could not be controlled for. Therefore, the long-term diabetic cats were not included in the statistical analysis. Another limitation is that for all cats, impaired glycemic control and the presence of insulin resistance was assessed indirectly and based on changes in serum glucose and fructosamine concentrations. Ideally, glycemic control and insulin resistance would be tested directly i.e., with glucose tolerance testing. However, given the logistical challenges and difficulties interpreting these tests in client owned cats, further investigation into the presence of insulin resistance in these cats was not performed.

Changes in plasma nesfatin-1 concentrations in humans have been associated with hypertension, cardiac disease, and behavioural or anxiety related conditions [56,57,60]. While no cats in our study had a history of these conditions, or any evidence on a routine clinical exam, further investigations into these conditions i.e., blood pressure measurement, echocardiography, or thorough behavioural assessment, was not performed. Finally, the nesfatin-1 assay we used for our feline plasma was designed for use with rat plasma or serum. The assay showed acceptable performance characteristics with our feline samples, though the variations in sample recovery at different concentrations would limit the usefulness of this assay for diagnostic purposes. Ideally a feline specific nesfatin-1 assay would be used, especially if plasma nesfatin-1 was to be measured for diagnostic purposes. A feline specific nesfatin-1 assay is not currently available, though it may be prudent to re-test these samples if and when and feline specific assay becomes available.

3.5.7 Future Studies

There are several directions that could be taken to further investigate links between plasma nesfatin-1 concentrations, adiposity, and glycemic control. Given the high nesfatin-1 concentrations seen in four individuals in the lean cat group, further investigation is indicated to find other factors unrelated to body condition or glycemic control that may influence nesfatin-1 concentrations, and also whether similarly high concentrations can be seen in individual

overweight and diabetic cats. A prospective study over a longer time frame in overweight and diabetic cats would be particularly beneficial, though logistically challenging. Lean cats with high nesfatin-1 concentrations could also be followed over time to see if any clinically relevant outcomes are associated with these high nesfatin-1 concentrations. For overweight cats, serial plasma nesfatin-1 measurements could be taken to see if they change over time. This would be of particular interest if any of the overweight cats became diabetic. Plasma nesfatin-1 concentrations could then be compared between cats that developed diabetes and those that didn't, to see if changes in nesfatin-1 concentrations do truly follow apparent changes in insulin resistance. In the newly diagnosed diabetic cats, it may also be beneficial to follow them over a longer time period. This, along with a larger sample size, would hopefully allow a comparison of diabetic cats who do and do not achieve remission, to determine if peripheral nesfatin-1 is predictive of successful treatment response. Finally, it may be useful to delineate the effects dietary modifications alone have on plasma nesfatin-1 concentrations. This could be assessed by comparing nesfatin-1 concentrations in lean and overweight, non-diabetic cats before and after instituting dietary modifications typically prescribed to diabetic cats.

3.6 Conclusions

In conclusion, this chapter investigated plasma nesfatin-1 concentrations in cats in relation to body condition and diabetes mellitus, with the results suggesting changes in plasma nesfatin-1 concentrations may be more closely related to alterations in glycemic control/ insulin resistance as opposed to changes in body condition. Nesfatin-1 levels in newly diagnosed diabetic cats may be altered in comparison to non-diabetic cats, though a larger sample size is required to definitively show this relationship. There may also be as yet unknown intrinsic or environmental factors that increase plasma nesfatin-1 levels, particular in lean cats, and this possibility also requires further investigation. In diabetic cats, plasma nesfatin-1 concentrations significantly decrease after short-term treatment, and this decrease may be more closely related to changes in glycemic control rather than changes in body weight, though further investigation is required to determine if this relationship is significant.

Summary and Conclusions

The overall goal of this thesis was to characterize tissue and plasma nesfatin-1 expression in cats, to better understand nesfatin-1's relationship to adiposity and glycemic control in this species. Our results, in conjunction with further research, will further our understanding of how nesfatin-1 may be implicated in the pathophysiology of obesity and diabetes mellitus in cats. This has the potential to lead to the discovery of additional diagnostic or therapeutic options to improve management of insulin resistance and diabetes mellitus.

The first chapter of the thesis explored the tissue mRNA expression of NUCB2/nesfatin-1, and how it differs between lean, overweight, and diabetic cats. Our results showed that NUCB2/nesfatin-1 mRNA was expressed in all tissues evaluated. mRNA expression is highest in the pancreas, which was a somewhat unexpected finding, and may point towards pancreatic nesfatin-1 having an autocrine mode of action in cats. Differences in NUCB2/nesfatin-1 expression between study groups were most pronounced in gastric and pancreatic tissue. Overweight cats had marginally decreased mRNA expression, whereas diabetic cats showed a biphasic mode of expression, with some diabetic cats having similar expression to lean healthy cats, and some having comparatively decreased expression. These changes were unexpected, as it was hypothesized that nesfatin-1 expression would be positively correlated with insulin resistance as a compensatory response to maintain glycemic control. Instead, these findings suggest that decreased nesfatin-1 expression in some overweight and diabetic cats may reflect islet cell injury and a diminished capacity to maintain normoglycemia.

The second chapter of this study explored plasma nesfatin-1 protein concentrations, and how they differ between lean, overweight, and diabetic cats. A rat nesfatin-1 ELISA was also validated for use with feline plasma. Both newly diagnosed and long-term treated diabetic cats had marginally increased plasma nesfatin-1 concentrations in comparison to lean and overweight non-diabetic cats, though these differences were not statistically significant. However, nesfatin-1 concentrations did decrease significantly in the newly diagnosed diabetic cats following short term treatment, which may suggest a connection between decreasing nesfatin-1 concentrations and improved glycemic control. The lack of a significant difference between nesfatin-1 levels in lean, overweight, and diabetic cats seen in this study may be due to a sub-population of lean cats with unexpectedly high plasma nesfatin-1 concentrations, which may point to additional, as yet

unknown factors that may increase nesfatin-1 concentrations. Differences in markers of adiposity and metabolism i.e., BCS, body weight, and cholesterol, were not associated with differences in plasma nesfatin-1 concentrations.

Interestingly, the results in plasma nesfatin-1 concentrations and NUCB2/nesfatin-1 mRNA expression were discordant with one another. As seen in chapter one, mRNA expression appears to trend downwards in overweight and diabetic cats in comparison to lean cats, whereas plasma nesfatin-1 concentrations appear to trend upwards in comparison to lean cats. It was thought that nesfatin-1 tissue expression would trend in the same direction as plasma concentrations. However, the data suggests that difference in tissue mRNA expression may not reflect differences in circulating nesfatin-1 protein concentrations. This discordance has been seen in a study in dogs, where differences in gastric mRNA expression did not correlate to changes in plasma nesfatin-1 [30]. It has also been documented in a study in rats, where differences in pancreatic nesfatin-1 protein expression did not correlate with differences in circulating nesfatin-1 concentrations [66]. It is also possible that nesfatin-1 expression in other tissues not assessed may play a significant role in the maintenance of plasma nesfatin-1 concentrations. These other tissues may include the liver, which plays an important role in compensating for the presence of insulin resistance in cats [2], and it cannot be entirely excluded that nesfatin-1 produced within the central nervous system has an effect on circulating nesfatin-1 concentrations. Finally, the NUCB2/nesfatin-1 mRNA expression documented, particularly in the pancreas, could reflect nesfatin-1 having an autocrine mode of action. In this way, changes in nesfatin-1 mRNA expression may reflect local changes within the pancreas, which do not affect circulating nesfatin-1 concentrations.

Future studies may involve further exploration into changes in mRNA expression in feline gastrointestinal tissues, in conjunction with analysis of nesfatin-1 protein expression and circulating nesfatin-1 levels, to obtain a more global picture of nesfatin-1 expression in cats. Further investigation is also indicated to see if significant differences do exist in plasma nesfatin-1 concentrations of lean, overweight, and diabetic cats, and to identify other conditions where plasma nesfatin-1 concentrations are altered. Longitudinal evaluation of plasma nesfatin-1 concentrations in cats over a longer time frame would be ideal, to further evaluate if nesfatin-1

concentrations change in individual cats as they develop increasing adiposity, insulin resistance, and eventually become clinically diabetic.

References

[1] Gottlieb S, Rand J. Managing feline diabetes: current perspectives. Vet Med Res Reports 2018;Volume 9:33–42. https://doi.org/10.2147/vmrr.s125619.

[2] Hoenig M. The cat as a model for human obesity and diabetes. J Diabetes Sci Technol 2012;6:525–33. https://doi.org/10.1177/193229681200600306.

[3] Scott-Moncrieff JC. Insulin Resistance in Cats. Vet Clin North Am - Small Anim Pract 2010;40:241–57. https://doi.org/10.1016/j.cvsm.2009.10.007.

[4] Gostelow R, Forcada Y, Graves T, Church D, Niessen S. Systematic review of feline diabetic remission: Separating fact from opinion. Vet J 2014;202:208–21. https://doi.org/10.1016/j.tvjl.2014.08.014.

[5] Mimura K, Mori A, Lee P, Ueda K, Oda H, Saeki K, et al. Impact of commercially available diabetic prescription diets on short- term postprandial serum glucose, insulin, triglyceride and free fatty acid concentrations of obese cats. J Vet Med Sci 2013;75:929–37. https://doi.org/10.1292/jvms.12-0310.

[6] Alt N, Kley S, Tschuor F, Zapf J, Reusch CE. Evaluation of IGF-1 levels in cats with transient and permanent diabetes mellitus. Res Vet Sci 2007;83:331–5. https://doi.org/10.1016/j.rvsc.2007.01.014.

[7] Marshall RD, Rand JS, Morton JM. Treatment of newly diagnosed diabetic cats with glargine insulin improves glycaemic control and results in higher probability of remission than protamine zinc and lente insulins. J Feline Med Surg 2009;11:683–91. https://doi.org/10.1016/j.jfms.2009.05.016.

[8] Zini E, Hafner M, Osto M, Franchini M, Ackermann M, Lutz TA, et al. P red i ctor s of Clinic al Re mi s s i o n i n Ca t s w i t h D i a b e t e s M e l l i t u s 2010:1314–21.

[9] Gottlieb S, Rand JS. Remission in Cats. Including Predictors and Risk Factors. Vet Clin North Am - Small Anim Pract 2013;43:245–9. https://doi.org/10.1016/j.cvsm.2013.01.001.

[10] Strage EM, Holst BS, Nilsson G, Jones B, Lilliehöök I. Validation of an enzyme-linked immunosorbent assay for measurement of feline serum insulin. Vet Clin Pathol 2012;41:518–28. https://doi.org/10.1111/j.1939-165x.2012.00476.x.

[11] Strage E. Biological variation of serum insulin concentrations in healthy cats. Acta Vet Scand 2015;57:O14. https://doi.org/10.1186/1751-0147-57-S1-O14.

[12] Altemani A, Alamri A, Ahmed M, Al Garbo M, Alharbi T, Al-Rasheed R, et al. Prediabetes and serum insulin levels. Int J Community Med Public Heal 2018;5:1684. https://doi.org/10.18203/2394-6040.ijcmph20181494.

[13] Oh-I S, Shimizu H, Satoh T, Okada S, Adachi S, Inoue K, et al. Identification of nesfatin-1 as a satiety molecule in the hypothalamus. Nature 2006;443:709–12. https://doi.org/10.1038/nature05162.

[14] Schalla MA, Stengel A. Current understanding of the role of nesfatin-1. J Endocr Soc 2018;2:1188–206. https://doi.org/10.1210/js.2018-00246.

[15] Shimizu H, Oh-I S, Hashimoto K, Nakata M, Yamamoto S, Yoshida N, et al. Peripheral administration of nesfatin-1 reduces food intake in mice: The leptin-independent mechanism. Endocrinology 2009;150:662–71. https://doi.org/10.1210/en.2008-0598.

[16] Lents CA, Barb CR, Hausman GJ, Nonneman D, Heidorn NL, Cisse RS, et al. Effects of nesfatin-1 on food intake and LH secretion in prepubertal gilts and genomic association of the porcine NUCB2 gene with growth traits1. Domest Anim Endocrinol 2013;45:89–97. https://doi.org/10.1016/j.domaniend.2013.06.002.

[17] Stengel A, Goebel M, Wang L, Rivier J, Kobelt P, Mönnikes H, et al. Central nesfatin-1 reduces dark-phase food intake and gastric emptying in rats: Differential role of corticotropin-releasing factor2 receptor. Endocrinology 2009;150:4911–9. https://doi.org/10.1210/en.2009-0578.

[18] Yosten GLC, Willis SK. The anorexigenic and hypertensive effects of nesfatin-1 are reversed by pretreatment with an oxytocin receptor antagonist.(Author abstract)(Report) 2010;298:R1642. https://doi.org/10.1152/ajpregu.00804.2009.

[19] Dore R, Levata L, Lehnert H, Schulz C. Nesfatin-1: Functions and physiology of a novel regulatory peptide. J Endocrinol 2017;232:R45–65. https://doi.org/10.1530/JOE-16-0361.

[20] Iwasaki Y, Nakabayashi H, Kakei M, Shimizu H, Mori M, Yada T. Nesfatin-1 evokes Ca 2+ signaling in isolated vagal afferent neurons via Ca 2+ influx through N-type channels. Biochem Biophys Res Commun 2009;390:958–62. https://doi.org/10.1016/j.bbrc.2009.10.085.

[21] Xia Z-F, Fritze DM, Li J-Y, Chai B, Zhang C, Zhang W, et al. Nesfatin-1 inhibits gastric acid secretion via a central vagal mechanism in rats.(Report)(Author abstract). Am J Physiol 2012;303:G570. https://doi.org/10.1152/ajpgi.00178.2012.

[22] Pałasz A, Krzystanek M, Worthington J, Czajkowska B, Kostro K, Wiaderkiewicz R, et al. Nesfatin-1, a unique regulatory neuropeptide of the brain. Neuropeptides 2012;46:105–12. https://doi.org/10.1016/j.npep.2011.12.002.

[23] Bonnet M, Djelloul M, Tillement V, Tardivel C, Mounien L, Trouslard J, et al. Central NUCB2/Nesfatin-1-Expressing Neurones Belong to the Hypothalamic-Brainstem Circuitry Activated by Hypoglycaemia. J Neuroendocrinol 2012;25. https://doi.org/10.1111/j.1365-2826.2012.02375.x.

[24] Pan W, Hsuchou H, Kastin AJ. Nesfatin-1 crosses the blood-brain barrier without saturation. Peptides 2007;28:2223–8. https://doi.org/10.1016/j.peptides.2007.09.005.

[25] Atsuchi K, Asakawa A, Ushikai M, Ataka K, Tsai M, Koyama K, et al. Centrally administered nesfatin-1 inhibits feeding behaviour and gastroduodenal motility in mice. Neuroreport 2010;21:1008–11. https://doi.org/10.1097/WNR.0b013e32833f7b96.

[26] Goebel M, Stengel A, Wang L, Taché Y. Central nesfatin-1 reduces the nocturnal food intake in mice by reducing meal size and increasing inter-meal intervals. Peptides 2011;32:36–43. https://doi.org/10.1016/j.peptides.2010.09.027.

[27] Mortazavi S, Gonzalez R, Ceddia R, Unniappan S. Long-term infusion of nesfatin-1 causes a sustained regulation of whole-body energy homeostasis of male Fischer 344 rats. Front Cell Dev Biol 2015;3:1–12. https://doi.org/10.3389/fcell.2015.00022.

[28] Stengel A, Goebel M, Yakubov I, Wang L, Witcher D, Coskun T, et al. Identification and characterization of nesfatin-1 immunoreactivity in endocrine cell types of the rat gastric oxyntic mucosa. Endocrinology 2009;150:232–8. https://doi.org/10.1210/en.2008-0747.

[29] Stengel A, Mori M, Taché Y. The role of nesfatin-1 in the regulation of food intake and body weight: Recent developments and future endeavors. Obes Rev 2013;14:859–70. https://doi.org/10.1111/obr.12063.

[30] Nozawa S, Kimura T, Kurishima M, Mimura K, Saeki K, Miki Y, et al. Analyses of a satiety factor NUCB2/nesfatin-1; gene expressions and modulation by different dietary components in dogs. J Vet Med Sci 2016;78:411–7. https://doi.org/10.1292/jvms.15-0255.

[31] Morton KA, Hargreaves L, Mortazavi S, Weber LP, Blanco AM, Unniappan S. Tissue-specific expression and circulating concentrations of nesfatin-1 in domestic animals. Domest Anim Endocrinol 2018;65:56–66. https://doi.org/10.1016/j.domaniend.2018.04.006.

[32] Macro JA, Dimaline R, Dockray GJ. Identification and expression of prohormone-converting enzymes in the rat stomach. Am J Physiol 1996;270:G87.

[33] Li QC, Wang HY, Chen X, Guan HZ, Jiang ZY. Fasting plasma levels of nesfatin-1 in patients with type 1 and type 2 diabetes mellitus and the nutrient-related fluctuation of nesfatin-1 level in normal humans. Regul Pept 2010;159:72–7. https://doi.org/10.1016/j.regpep.2009.11.003.

[34] Gonkowski S, Rychlik A, Nowicki M, Nieradka R, Bulc M, Całka J. A population of nesfatin 1-like immunoreactive (LI) cells in the mucosal layer of the canine digestive tract. Res Vet Sci 2012;93:1119–21. https://doi.org/10.1016/j.rvsc.2012.06.002.

[35] Varricchio E, Russolillo MG, Russo F, Lombardi V, Paolucci M, Maruccio L. Expression and immunohistochemical detection of Nesfatin-1 in the gastrointestinal tract of Casertana pig. Acta Histochem 2014;116:583–7. https://doi.org/10.1016/j.acthis.2013.11.006.

[36] Hatef A, Shajan S, Unniappan S. Nutrient status modulates the expression of nesfatin-1 encoding nucleobindin 2A and 2B mRNAs in zebrafish gut, liver and brain. Gen Comp

Endocrinol 2015;215:51–60. https://doi.org/10.1016/j.ygcen.2014.09.009.

[37] Li Z, Gao L, Tang H, Yin Y, Xiang X, Li Y, et al. Peripheral Effects of Nesfatin-1 on Glucose Homeostasis. PLoS One 2013;8. https://doi.org/10.1371/journal.pone.0071513.

[38] Prinz P, Stengel A. Nesfatin-1: current status as a peripheral hormone and future prospects. Curr Opin Pharmacol 2016;31:19–24. https://doi.org/10.1016/j.coph.2016.08.011.

[39] Zhang X, Wang S, Chen H, Tang N, Qi J, Wu Y, et al. The inhibitory effect of NUCB2/nesfatin-1 on appetite regulation of Siberian sturgeon (Acipenser baerii Brandt). Horm Behav 2018;103:111–20. https://doi.org/10.1016/j.yhbeh.2018.06.008.

[40] Price TO, Samson WK, Niehoff ML, Banks WA. Permeability of the blood-brain barrier to a novel satiety molecule nesfatin-1. Peptides 2007;28:2372–81. https://doi.org/10.1016/j.peptides.2007.10.008.

[41] Ge JF, Xu YY, Qin G, Pan XY, Cheng JQ, Chen FH. Nesfatin-1, a potent anorexic agent, decreases exploration and induces anxiety-like behavior in rats without altering learning or memory. Brain Res 2015;1629:171–81. https://doi.org/10.1016/j.brainres.2015.10.027.

[42] Yang M, Zhang Z, Wang C, Li K, Li S, Boden G, et al. Nesfatin-1 action in the brain increases insulin sensitivity through Akt/AMPK/TORC2 pathway in diet-induced insulin resistance. Diabetes 2012;61:1959. https://doi.org/10.2337/db11-1755.

[43] Dong J, Xu H, Wang P, Cai G, Song H, Wang C, et al. Nesfatin-1 Stimulates Fatty-Acid Oxidation by Activating AMP-Activated Protein Kinase in STZ-Induced Type 2 Diabetic Mice.(Research Article). PLoS One 2013;8:e83397. https://doi.org/10.1371/journal.pone.0083397.

[44] Yin Y, Li Z, Gao L, Li Y, Zhao J, Zhang W. AMPK-dependent modulation of hepatic lipid metabolism by nesfatin-1. Mol Cell Endocrinol 2015;417:20–6. https://doi.org/10.1016/j.mce.2015.09.006.

[45] Su Y, Zhang J, Tang Y, Bi F, Liu JN. The novel function of nesfatin-1: Anti-hyperglycemia. Biochem Biophys Res Commun 2010;391:1039–42.

https://doi.org/10.1016/j.bbrc.2009.12.014.

[46] Tan BK, Hallschmid M, Kern W, Lehnert H, Randeva HS. Decreased cerebrospinal fluid/plasma ratio of the novel satiety molecule, nesfatin-1/NUCB-2, in obese humans: Evidence of nesfatin-1/NUCB-2 resistance and implications for obesity treatment. J Clin Endocrinol Metab 2011;96:669–73. https://doi.org/10.1210/jc.2010-1782.

[47] Zoran DL. Obesity in Dogs and Cats: A Metabolic and Endocrine Disorder. Vet Clin North Am - Small Anim Pract 2010;40:221–39. https://doi.org/10.1016/j.cvsm.2009.10.009.

[48] Klempel MC, Varady KA. Reliability of leptin, but not adiponectin, as a biomarker for diet-induced weight loss in humans. Nutr Rev 2011;69:145–54. https://doi.org/10.1111/j.1753-4887.2011.00373.x.

[49] Williams MC, McMillan CJ, Snead ER, Takada K, Chelikani PK. Association of circulating adipokine concentrations with indices of adiposity and sex in healthy, adult client owned cats. BMC Vet Res 2019;15:1–11. https://doi.org/10.1186/s12917-019-2080-9.

[50] Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE, et al. Hypoadiponectinemia in obesity and type 2 diabetes: Close association with insulin resistance and hyperinsulinemia. J Clin Endocrinol Metab 2001;86:1930–5. https://doi.org/10.1210/jcem.86.5.7463.

[51] Gonzalez R, Reingold BK, Gao X, Gaidhu MP, Tsushima RG, Unniappan S. Nesfatin-1 exerts a direct, glucose-dependent insulinotropic action on mouse islet β- and MIN6 cells. J Endocrinol 2011;208. https://doi.org/10.1530/JOE-10-0492.

[52] Zhang Z, Li L, Yang M, Liu H, Boden G, Yang G. Increased plasma levels of nesfatin-1 in patients with newly diagnosed type 2 diabetes mellitus. Exp Clin Endocrinol Diabetes 2012;120:91. https://doi.org/10.1055/s-0031-1286339.

[53] Guo Y, Liao Y, Fang G, Dong J, Li Z. Increased nucleobindin-2 (NUCB2) transcriptional activity links the regulation of insulin sensitivity in Type 2 diabetes mellitus. J Endocrinol

Invest 2013;36:883–8. https://doi.org/10.3275/9000.

[54] Akin Ş, Gülçiçek NE, Yazgan Aksoy D, Karakaya J, Usman A. Increased serum nesfatin-1 levels in patients with impaired glucose tolerance. Turkish J Endocrinol Metab 2017;21:65–7. https://doi.org/10.25179/tjem.2017-56543.

[55] Zhao Y, Ma X, Wang Q, Zhou Y, Zhang Y, Wu L, et al. Nesfatin-1 correlates with hypertension in overweight or obese Han Chinese population. Clin Exp Hypertens 2015;37:51–6. https://doi.org/10.3109/10641963.2014.897722.

[56] Kuyumcu A. The relationship between nesfatin-1 and carotid artery stenosis. Scand Cardiovasc J 2018;52:328–34. https://doi.org/10.1080/14017431.2018.1547840.

[57] Dai H, Li X, He T, Wang Y, Wang Z, Wang S, et al. Decreased plasma nesfatin-1 levels in patients with acute myocardial infarction. Peptides 2013;46:167–71. https://doi.org/10.1016/j.peptides.2013.06.006.

[58] Mazza R, Gattuso A, Filice M, Cantafio P, Cerra MC, Angelone T, et al. Nesfatin-1 as a new positive inotrope in the goldfish (Carassius auratus) heart. Gen Comp Endocrinol 2015;224:160–7. https://doi.org/10.1016/j.ygcen.2015.08.003.

[59] Stengel A, Goebel M, Taché Y. Nesfatin-1: A novel inhibitory regulator of food intake and body weight. Obes Rev 2011;12:261–71. https://doi.org/10.1111/j.1467-789X.2010.00770.x.

[60] Xiao MM, Li JB, Jiang LL, Shao H, Wang BL. Plasma nesfatin-1 level is associated with severity of depression in Chinese depressive patients. BMC Psychiatry 2018;18:1–7. https://doi.org/10.1186/s12888-018-1672-4.

[61] Yang M, Zhang Z, Wang C, Li K, Li S, Boden G, et al. Nesfatin-1 action in the brain increases insulin sensitivity through Akt/AMPK/TORC2 pathway in diet-induced insulin resistance. Diabetes 2012;61:1959–68. https://doi.org/10.2337/db11-1755.

[62] Algul S, Ozkan Y, Ozcelik O. Serum nesfatin-1 levels in patients with different glucose tolerance levels. Physiol Res 2016;65:979–85. https://doi.org/10.33549/physiolres.933186.

[63] Zhai T, Li SZ, Fan XT, Tian Z, Lu XQ, Dong J. Circulating Nesfatin-1 Levels and Type 2 Diabetes: A Systematic Review and Meta-Analysis. J Diabetes Res 2017;2017. https://doi.org/10.1155/2017/7687098.

[64] Kessler Y, Helfer-Hungerbuehler AK, Cattori V, Meli ML, Zellweger B, Ossent P, et al. Quantitative TaqMan®real-time PCR assays for gene expression normalisation in feline tissues. BMC Mol Biol 2009;10. https://doi.org/10.1186/1471-2199-10-106.

[65] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 2001;25:402–8. https://doi.org/10.1006/meth.2001.1262.

[66] Foo KS, Brauner H, Östenson CG, Broberger C. Nucleobindin-2/nesfatin in the endocrine pancreas: Distribution and relationship to glycaemic state. J Endocrinol 2010;204:255–63. https://doi.org/10.1677/JOE-09-0254.

[67] Laflamme D. Development and validation of a body condition score system for cats: A clinical tool. Feline Pract 1997;25:13–8.

[68] Saracevic A, Nikolac N, Simundic AM. The evaluation and comparison of consecutive high speed centrifugation and LipoClear® reagent for lipemia removal. Clin Biochem 2014;47:309–14. https://doi.org/10.1016/j.clinbiochem.2014.01.001.

[69] Appleton D, Rand J, Sunvold G. Insulin sensitivity decreases with obesity, and lean cats with low insulin sensitivity are at greatest risk of glucose intolerance with weight gain. J Feline Med Surg 2001;3:211–28. https://doi.org/10.1053/jfms.2001.0138.

[70] Sallander M, Eliasson J, Hedhammar A. Prevalence and risk factors for the development of diabetes mellitus in Swedish cats. Acta Vet Scand 2012;54:61. https://doi.org/10.1186/1751-0147-54-61.

[71] Gottlieb S, Rand J, Anderson ST, Morton JM, Dias DA, Boughton BA, et al. Metabolic Profiling of Diabetic Cats in Remission. Front Vet Sci 2020;7. https://doi.org/10.3389/fvets.2020.00218.

[72] Hewson-Hughes AK, Gilham MS, Upton S, Colyer A, Butterwick R, Miller AT.

Postprandial glucose and insulin profiles following a glucose-loaded meal in cats and dogs. Br J Nutr 2011;106 Suppl:S101. https://doi.org/10.1017/S0007114511000857.

Appendix A: Supplemental Tables and Figures

Lean Cats	Tissues Available	Age	Sex	Weight (kg)	BCS (/9)	Breed	Blood Glucose (mmol/L)	Other Comments
LC1	Stomach, Pancreas, Skeletal Muscle, Abdominal Adipose	3	M Entire	-	4/9	DSH	7.9	No gross lesions
LC2	Stomach, Pancreas Skeletal Muscle	3	M Entire	-	4/9	DSH	6.1	No visual fat pad No gross lesions
LC3	Stomach, Pancreas, Skeletal Muscle, Abdominal Adipose	17	FS	3.8	5/9	DSH	7.7	Mildly increased urea Pancreas appeared nodular
LC4	Stomach, Skeletal Muscle	7	MN	5.7	4/9	DMH	4.1	No abdominal adipose or pancreas No gross lesions
Overweight Cats								
OC1	Stomach, Pancreas, Skeletal Muscle, Abdominal Adipose	7	MN	6.97	8/9	DSH	5.3	No gross lesions identified Diarrhoea in colon
OC2	Stomach, Pancreas, Skeletal Muscle, Abdominal Adipose	7	MN	9.46	9/9	DSH	3.9	Mild ocular discharge No gross lesions identified
OC3	Stomach, Pancreas, Skeletal Muscle, Abdominal Adipose	12	MN	5.09	6/9	Siamese	7.0	Ear margins – scar tissue/ excoriations Dental disease. Very nodular pancreas with a fluid filled cyst in the right limb

Diabetic Cats								
DC1	Stomach, Pancreas, Skeletal Muscle, Abdominal Adipose	6-8	F Entire	2.73	1/9	DMH	34	Untreated Diabetic. FeLV Positive Moderately increased serum urea Stray cat, history unknown
DC2	Stomach, Pancreas, Skeletal Muscle, Abdominal Adipose	8	MN	5.46	6/9	DSH	28.9	Treated for 3 weeks Diffusely nodular pancreas (small pale nodules), Pale liver
DC3	Stomach, Pancreas, Skeletal Muscle, Abdominal Adipose	10	FS	3.67	4/9	DSH	26.8	Untreated Diabetic No gross lesions
DC4	Stomach, Pancreas, Skeletal Muscle, Abdominal Adipose	7	MN	8.83	6/9	Maine Coon	27.3	Uncontrolled Diabetic – Treated for 2 months, then euthansed due to DKA (Ketones 5.1), increased serum urea Micro-abscess in the left limb of the pancreas, Fatty liver
DC5	Stomach, Pancreas, Skeletal Muscle, Abdominal Adipose	7	FS	4.2	4/9	Siamese X	13.6	Uncontrolled Diabetic – Treated for 2-3 months, then euthansed due to DKA (Ketones 5.3) Icteric, moderate dental disease Diffusely nodular pancreas Fatty liver

Supplemental Table 2.1: Physical exam, necropsy, and historical findings for all cats (n = 12) enrolled for tissue collection and NUCB2/nesfatin-1 mRNA expression (Chapter 1).

Primer Name	Direction	Sequence	Amplicon Size (bp)
NUCB2	Forward (5' – 3')	AGGCAAGAAGTGGCAAGGTT	175
	Reverse (5' – 3')	GATCACTTGTGGCCGCTTTG	
ß-actin	Forward	CGCAAGTACTCCGTGTGGAT	195
	Reverse	TTGCCAGGGCAAACCTAGAC	
RPS7	Forward	GTCCCAGAAGCCGCACTTT	74
	Reverse	CACAATCTCGCTCGGGAAAA	

Supplemental Table 2.2: Primer sequences for NUCB2/nesfatin-1 and the two reference genes, ß-actin and Ribosomal Protein 7 (RPS7), used for evaluating relative tissue NUCB2/nesfatin-1 expression by one-step RT-PCR

	2^(-▲▲ct) Relative NUCB2/nesfatin-1 mRNA Expression			
	Stomach	**Pancreas**	**Muscle**	**Abdominal Fat**
LC1	1.21	93.05	0.86	0.62
LC2	1.27	109.9	0.45	NA
LC3	2.22	128.89	1.8	3.39
LC4	0.8	NA	0.32	NA
OC1	0.62	81.01	1.03	1.38
OC2	1.35	28.25	0.74	2.22
OC3	1.07	83.87	0.65	1.06
DC1	0.51	33.36	0.4	0.71
DC2	0.43	11.08	0.74	1.44
DC3	1.41	147.03	0.68	0.99
DC4	0.72	16	0.71	1.37
DC5	1.87	99.73	0.68	2.16

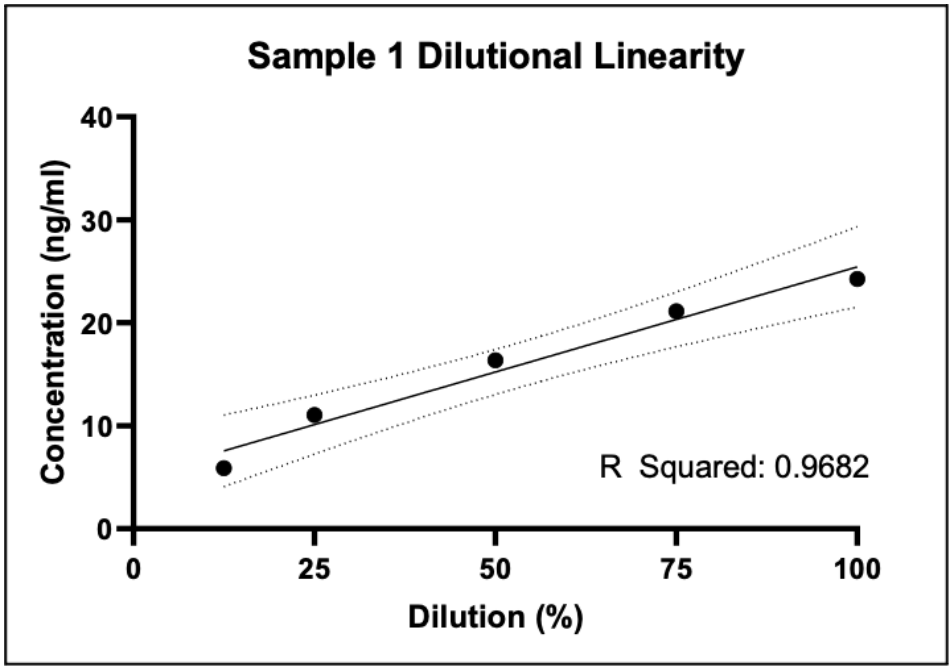
Supplemental Table 2.3: Relative NUCB2/nesfatin-1 tissue expression of gastric, pancreatic, skeletal muscle, and abdominal fat of individual cats in comparison to average gastric expression from all 12 cats

	Measured Concentration (ng/ml)	Calculated Concentration (ng/ml)	Sample Recovery
Sample 1	17.18	-	-
Sample 2	13.63	-	-
80% S1 + 20% S2	17.32	16.58	104%
50% S1 + 50% S2	17.03	15.41	111%
20% S1 + 80% S2	14.7	14.23	103%

Supplemental Table 3.1: Individual modified spiked recovery test using two feline plasma samples mixed in three separate ratios. Measured concentrations compared to calculated concentrations

Sample Dilution (Unknown 1)	Measured Concentration (ng/ml)	Expected Concentration (ng/ml)	Recovery (%)
1:1	24.24	-	-
3:4	21.11	18.18	116
1:2	16.35	12.12	135
1:4	11.05	6.06	182
1:8	5.88	3.03	194

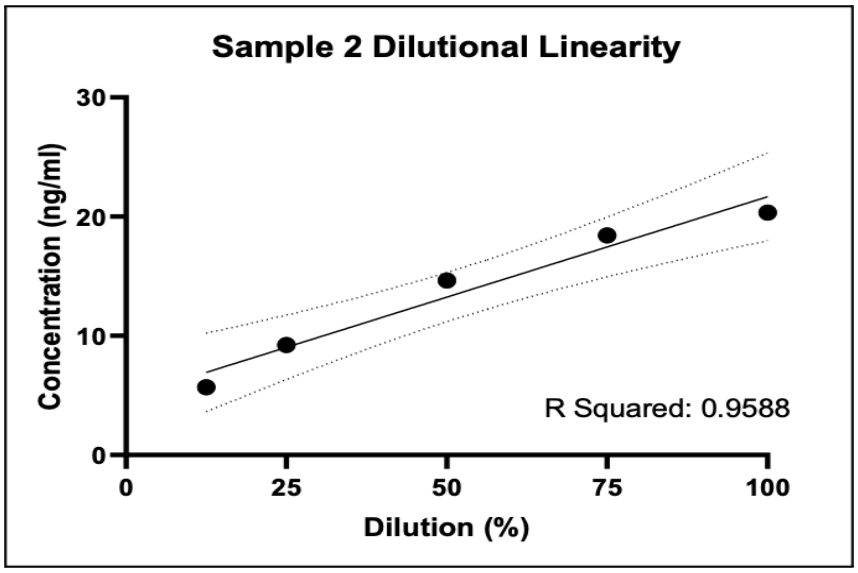
Supplemental Table 3.2: Serial Dilution of Unknown Sample 1, measured concentrations under dilution compared to expected calculated concentration



Supplemental Figure 3.1: Serial Dilution of Unknown Sample 1, 3:4, 1:2, 1:4, 1:8 dilutions

Sample Dilution (Unknown 2)	Measured Concentration (ng/ml)	Expected Concentration (ng/ml)	Recovery (%)
1:1	20.34	-	-
3:4	18.43	15.26	121
1:2	14.65	10.17	144
1:4	9.22	5.09	181
1:8	5.69	2.54	224

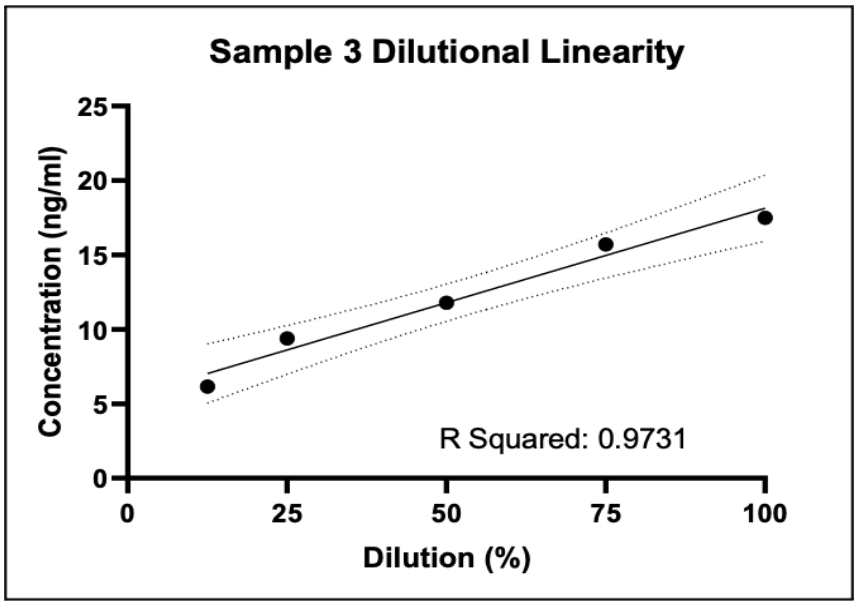
Supplemental Table 3.3: Serial Dilution of Unknown Sample 2, measured concentrations under dilution compared to expected calculated concentration



Supplemental Figure 3.2: Serial Dilution of Unknown Sample 1, 3:4, 1:2, 1:4, 1:8 dilutions

Sample Dilution (Unknown 3)	Measured Concentration (ng/ml)	Expected Concentration (ng/ml)	Recovery (%)
1:1	17.5	-	-
3:4	15.7	13.13	120
1:2	11.79	8.75	135
1:4	9.38	4.38	214
1:8	6.16	2.19	281

Supplemental Table 3.4: Serial Dilution of Unknown Sample 3, measured concentrations under dilution compared to expected calculated concentration



Supplemental Figure 3.3: Serial Dilution of Unknown Sample 1, 3:4, 1:2, 1:4, 1:8 dilutions

Patient Characteristics	
Group	Cats were allocated into three groups according to their body condition score and diabetic status. The first group consists of lean, non-diabetic cats (body condition score 4-5/9). The second group consists of overweight, non-diabetic cats (body condition score 7-9/9). The third group consists of diabetic cats, of any body condition.
Body Condition	Cats were allocated into two groups. Cats were defined as being lean, with a body condition score of less than six, and overweight, cats having a body condition of greater than six. One diabetic cat had a body condition score of six, and was placed in the overweight group
Age	Cats were allocated into three groups. Groups were defined as being less than five years of age, five to nine years of age, and greater than 9 years of age
Diabetic Status	Cats were allocated into two groups, categorised as being either non-diabetic or diabetic, regardless of body condition
Sex	Cats were sorted into two groups according to their sex (male or female)

Supplemental Table 3.5: Definitions of categorical variables used for statistical analysis of plasma nesfatin-1 concentrations in lean, overweight, and newly diagnosed diabetic cats

Variables	Coefficient	R-Squared	95% CI	Prob > F
Nesfatin with Fructosamine	-0.01	0.0001	-0.5 – 0.47	0.96
Nesfatin with Cholesterol	0.01	0.01	-0.29 – 0.06	0.51
Nesfatin with Weight	-0.3	0.08	-0.54 – 0.06	0.1
Nesfatin with Glucose	-0.01	0.00	-0.7 – 0.68	0.98

Supplemental Table 3.6: Association between Plasma Nesfatin-1 and selected continuous variables in 13 lean, 13 overweight, and 8 newly diagnosed diabetic cats (n = 34 cats).

Variables	Coefficient	R-Squared	95% CI	Prob > F
Nesfatin with Fructosamine	0.27	0.22	0.07 – 0.46	0.01
Nesfatin with Cholesterol	0.06	0.04	-0.05 – 0.16	0.28
Nesfatin with Weight	-0.07	0.02	-0.24 – 0.11	0.44
Nesfatin with Glucose	0.32	0.15	0.03 – 0.61	0.03

Supplemental Table 3.7: Association between Plasma Nesfatin-1 and selected continuous variables in 9 lean, 13 overweight, and 8 newly diagnosed diabetic cats (n = 30 cats). The four highest plasma nesfatin-1 concentrations have been removed from this analysis

Variables	Coefficient	R-Squared	95% CI	Prob > F
ΔWeight with ΔGlucose	0.20	0.30	-0.10 – 0.50	0.16
ΔWeight with ΔFructosamine	0.38	0.92	0.25 – 0.52	<0.01
ΔGlucose with ΔFructosamine	0.61	0.38	-0.28 – 1.5	0.14

Supplemental Table 3.8: Association between changes in weight, glucose, and fructosamine, independent of changes in nesfatin-1 between diabetic cats at their initial presentation and following four weeks of treatment

Multivariate Regression Model		R^2	P > F
		0.59	0.17
Variables	**Prob >F**	**Coefficient**	**95% CI**
ΔGlucose	0.14	0.59	-1.5 – 0.3
ΔFructosamine	0.93	0.03	-0.91 – 0.85

Supplemental Table 3.9: Multivariate model assessing changes in glucose in relation to changes in plasma nesfatin-1 in diabetic cats at their initial presentation and following four weeks of treatment. Fructosamine is added as a confounding variable.

Appendix B: Owner Consent and Enrolment Forms

Consent Form For Animal Owners – Nesfatin-1 in Lean and Obese Healthy Cats

You have been invited to enter _____ (pet's name) in a research project entitled **"Evaluation of nesfatin-1 in lean, obese and diabetic cats"**. Please read this form carefully, and feel free to ask questions you might have.

Researcher(s):

Dr. Melissa Meachem: Department of Veterinary Pathology, Western College of Veterinary Medicine (WCVM). Primary Investigator; melissa.meachem@usask.ca, 306-966-2688
Dr. Peter Toh: Graduate Student, Department of Veterinary Pathology, Western College of Veterinary Medicine. Co-Primary Investigator; peter.toh@usask.ca, 306-966-7309
Dr. Elizabeth Snead: Small Animal Clinical Sciences, Western College of Veterinary Medicine (WCVM); liz.snead@usask.ca, 306-966-7068
Dr. Chantal McMillan: Department of Clinical and Diagnostic Sciences, University of Calgary Faculty of Veterinary Medicine, +1 (403) 210-6002

Funding Source(s): Companion Animal Health Fund, Western College of Veterinary Medicine

Purpose and Objective of the Study:

Similar to humans, there has been a major increase in the incidence of diabetes in pet cats, which is often associated with obesity related changes in insulin sensitivity. Management of these cats could be greatly improved if we could more accurately predict and monitor how individual cats will respond to treatment, or even identify a 'pre-diabetic state,' in which cats have some degree of insulin resistance but are not yet clinically diabetic. At this point in time, there are no tests available to accomplish this. Nesfatin-1 is a recently discovered hormone that plays a role in regulating food intake, blood sugar and insulin levels. Studies in humans have shown that increases in nesfatin-1 are correlated with other signs of insulin resistance in obese and diabetic people, and that nesfatin-1 levels decrease following treatment of type 2 diabetes.

Description of the Procedures: If your pet participates in this study, we will ask you a few questions about their medical history. Your pet will have a physical exam, and a single small

blood and urine sample will also be taken to look at a few health parameters in addition to nesfatin-1 levels. The urine sample will be collected by placing a needle through the abdominal wall into the urinary bladder (cystocentesis) using ultrasound guidance. The method of blood and urine collection will be performed according to routine VMC guidelines, and are standard procedures for collecting blood and urine samples in small animal patients. However, we will be collecting a slightly larger blood sample than routine for the purposes of our study. The blood and urine sampling should take about 30 minutes and will take place at the Veterinary Medical Centre.

Knowledge Transfer: The results of this study will be analyzed and reported in a manuscript with the goal of being published in a scientific journal. Results of blood work to assess general health will be communicated to you.

Potential Benefits: Diabetes mellitus is a common disease in cats that requires both financial and time commitments from owners. This study aims to provide new information that will not only help us to further understand the development of this condition, but also potentially help identify at risk individuals. In addition, by choosing to participate, your cat will receive a free physical exam by a veterinarian, as well as blood and urine work that will allow you to have greater knowledge of your cat's overall health.

Potential Risks and Discomforts: While small, there is a risk of your pet developing bruising at the site of the blood collection. Cystocentesis to collect a urine sample is a quick and simple procedure and risks will be minimized by use of ultrasound guidance to locate the bladder. All researchers are experienced at blood and urine collection and will be using appropriate techniques and materials to minimize these risks.

Financial Implications: There will be no cost to you for entering your animal(s) in this study. You will not be charged for any of the procedures performed solely for the study's purposes, including blood work and urinalysis to assess general health.

Confidentiality: While absolute confidentiality cannot be guaranteed, every effort will be made to ensure that the information collected for this study is kept entirely confidential. Each cat will be deidentified with an alphanumeric code for the purpose of the study. Your name or that of

your animal(s) will not be attached to any information nor mentioned in any study report, nor be made available to anyone except the research team. Results of this study are intended for publication in scientific journals and presentation at related conferences and workshops, but your identity or that of your animal(s) will not be revealed.

Data Storage: All research materials will be stored in a **secured location under lock- and-key for a period of 5 years post publication minimum as per the U of S Responsible Conduct of Research Policy, see:** http://www.usask.ca/university_secretary/policies/research/8_25.php).

Voluntary Participation: Your animal's participation is voluntary, and you may withdraw your animal(s) from the research project for any reason, at any time, without penalty of any sort. If you do not wish to participate, you do not have to provide any reason for your decision, nor will you lose the benefit of any veterinary care your animal(s) is receiving. If you withdraw your animal(s) from the research project any data collected about him or her during their enrollment in the study will be retained for analysis.

Questions: If you have any questions concerning the research project, please feel free to ask at any point; you can contact the primary investigators (Dr. Meachem or Dr. Toh) by phone or e-mail using the contact details listed above. This research project has been approved on ethical grounds by the University of Saskatchewan Animal Research Ethics Board. Any questions regarding the ethical conduct of this research may be addressed to that committee through the Research Ethics Office (306-966- 7928). Participants from outside of Saskatoon may call toll free 1-888-966-2975.

Consent to Participate: Please read and initial the following statements to signify your agreement and sign below.

_____ I have read or have had the consent form read to me and I understand the consent form.

_____ I have had an opportunity to ask questions and my/our questions have been answered.

_____ I freely consent to entering my animal(s) in this study.

_____ I have been told that a signed and dated copy of this Consent Form will be given to me for my records.

_____ I am at least 18 years of age and am the legal owner of the animal(s) or am authorized to make decisions regarding this(these) animal(s) on the owner's behalf.

_____ Signature of Owner or Agent

_____ Date

_____Signature of Individual conducting the Consent Process

_____ Date

Consent Form For Animal Owners – Feline Diabetics

You have been invited to enter _____(pet's name) in a research project entitled **"Evaluation of nesfatin-1 in lean, obese and diabetic cats"**. Please read this form carefully, and feel free to ask questions you might have.

Researcher(s):

Dr. Melissa Meachem: Department of Veterinary Pathology, Western College of Veterinary Medicine (WCVM). Primary Investigator; melissa.meachem@usask.ca, 306-966-2688
Dr. Peter Toh: Graduate Student, Department of Veterinary Pathology, Western College of Veterinary Medicine. Co-Primary Investigator; peter.toh@usask.ca, 306-966-7309
Dr. Elisabeth Snead: Small Animal Clinical Sciences, Western College of Veterinary Medicine (WCVM); liz.snead@usask.ca, 306-966-7068
Dr. Chantal McMillan: Department of Clinical and Diagnostic Sciences, University of Calgary Faculty of Veterinary Medicine, +1 (403) 210-6002

Funding Source(s):

Companion Animal Health Fund, Western College of Veterinary Medicine

Purpose and Objective of the Study:

Similar to humans, there has been a major increase in the incidence of diabetes in pet cats, which is often associated with obesity related changes in insulin sensitivity and insulin function. Management of these cats could be greatly improved if we could more accurately predict and monitor how individual cats will respond to treatment. At this point in time, there are no tests available to accomplish this. Nesfatin-1 is a recently discovered hormone that plays a role in regulating food intake, blood sugar and insulin levels. Studies in humans have shown that increases in nesfatin-1 are correlated with other signs of insulin resistance in obese and diabetic people, and that nesfatin-1 levels decrease with treatment of type 2 diabetes. Recent research has shown that nesfatin-1 can be measured in cats using a commercially available assay. Given feline diabetes shares many similarities with type 2 diabetes in humans, we predict that nesfatin-1 will

have similar effects and will be a useful marker in predicting or monitoring response to treatment.

Description of the Procedures: If your pet participates in this study, we will ask you a few questions about their medical history. Your pet will have a physical exam and a blood and urine sample will also be taken to look at a few health parameters and at nesfatin-1 levels, prior to starting diabetic treatment.

The urine sample will be collected by placing a needle through the abdominal wall into the urinary bladder (cystocentesis) using ultrasound guidance. The blood and urine sampling should take about 30 minutes and will take place at the Veterinary Medical Centre. The method of blood and urine collection will be performed according to routine VMC guidelines and are standard procedures for collecting blood and urine samples in small animal patients. However, we will be collecting a slightly larger blood sample than routine for the purposes of our study.

Following the initial blood work, your cat will begin diabetic treatment (insulin injections and a low carbohydrate diet), and additional blood work will be taken at 4 weeks into their treatment, to assess nesfatin-1 levels and a few other health parameters. At this appointment, your cat will also have glucose monitoring device placed, which will be attached to skin on the backs of their neck. The devices will stay in place for up to 14 days at a time, and you will be given a device to record the results electronically, you will need to record the results using the device at least every 8 hours. Overall you will be required to attend 2 appointment 4 weeks apart, both of which will take around 30-40 minutes each, and you will also be required to measure blood glucose readings at home electronically for up to 14 days after placement of the monitoring device.

Knowledge Transfer: The results of this study will be analyzed and reported in a manuscript with the goal of being published in a scientific journal. Results of blood work to assess general health parameters and information derived from an implanted blood glucose device will be communicated to you.

Potential Benefits: Diabetes mellitus is a common disease in cats that requires both financial and time commitments from owners. This study aims to provide new information that will not only help understand the development of this condition, but also potentially help identify at risk

individuals. In addition, by choosing to participate, your cat will receive a free physical exam by veterinarians, blood work and urinalysis that will assess their general health, a device that allows monitoring of blood glucose at home, and food specially formulated for diabetic cats. This will enable you to understand more about the overall health of your cat, as well as ensuring your cat is receiving optimal management for the diabetes.

Potential Risks and Discomforts: While small, there is a risk of your pet developing bruising at the site of the blood collection. Cystocentesis to collect a urine sample is a quick and simple procedure and risks will be minimized by use of ultrasound guidance to locate the bladder. All researchers are experienced at blood and urine collection and will be using appropriate techniques and materials to minimize these risks. After placement of the glucose monitoring device, your cat will also be required to wear a soft protective collar while it is attached

Financial Implications: There will be no cost to you for entering your animal(s) in this study. You will not be charged for any of the procedures performed solely for the study's purposes. You will be reimbursed for the initial blood work, and the blood work at the scheduled re-check appointment 4 weeks later. **There is no cost associated with use of the blood glucose measuring device, and at both appointments you will receive dry food and/or canned food (sufficient for 4 weeks) courtesy of Purina®. If unforeseen circumstances arise and your cat passes away or is withdrawn from the study, you will be reimbursed for the blood work during the time which your cat was part of the study.** All unrelated or additional costs for diagnosis, management and treatment of your animal(s) outside of these scheduled appointments are your responsibility. You will receive no additional financial reimbursement for entering your animal(s) in this study.

Confidentiality: While absolute confidentiality cannot be guaranteed, every effort will be made to ensure that the information collected for this study is kept entirely confidential. Each cat will be deidentified with an alphanumeric code for the purpose of the study. Your name or that of your animal(s) will not be attached to any information nor mentioned in any study report, nor be made available to anyone except the research team. Results of this study are intended for publication in scientific journals and presentation at related conferences and workshops, but your identity or that of your animal(s) will not be revealed.

Data Storage: All research materials will be stored in a **secured location under lock- and-key for a period of 5 years post publication minimum as per the U of S Responsible Conduct of Research Policy, see:** http://www.usask.ca/university_secretary/policies/research/8_25.php).

Voluntary Participation: Your animal's participation is voluntary, and you may withdraw your animal(s) from the research project for any reason, at any time, without penalty of any sort. If you do not wish to participate, you do not have to provide any reason for your decision, nor will you lose the benefit of any veterinary care your animal(s) is receiving. If you withdraw your animal(s) from the research project any data collected about him or her during their enrollment in the study will be retained for analysis.

Questions: If you have any questions concerning the study protocol, please feel free to ask at any point; you can contact the primary investigators (Dr. Meachem or Dr. Toh). **If you have concerns about the health of your cat, or questions regarding the management of your diabetic cat during the study, then you can contact Dr. Snead.** Please use the phone numbers or e-mail addresses provided above. This research project has been approved on ethical grounds by the University of Saskatchewan Animal Research Ethics Board. Any questions regarding the ethical conduct of this research may be addressed to that committee through the Research Ethics Office (306-966- 7928). Participants from outside of Saskatoon may call toll free 1-888-966-2975.

Consent to Participate: Please read and initial the following statements to signify your agreement and sign below.

_____ I have read or have had the consent form read to me and I understand the consent form.

_____ I have had an opportunity to ask questions and my/our questions have been answered.

_____ I freely consent to entering my animal(s) in this study.

_____ I have been told that a signed and dated copy of this Consent Form will be given to me for my records.

_____ I am at least 18 years of age and am the legal owner of the animal(s) or am authorized to make decisions regarding this(these) animal(s) on the owner's behalf.

_____ Signature of Owner or Agent

_____ Date

_____Signature of Individual conducting the Consent Process

_____ Date

Consent Form for Animal Owners – Nesfatin-1 in Long Term Diabetic Cats

You have been invited to enter _____ (pet's name) in a research project entitled

"**Evaluation of nesfatin-1 in lean, obese and diabetic cats**". Please read this form carefully, and feel free to ask questions you might have.

Researcher(s):

Dr. Melissa Meachem: Department of Veterinary Pathology, Western College of Veterinary Medicine (WCVM). Primary Investigator; melissa.meachem@usask.ca, 306-966-2688

Dr. Peter Toh: Graduate Student, Department of Veterinary Pathology, Western College of Veterinary Medicine. Co-Primary Investigator; peter.toh@usask.ca, 306-966-7309

Dr. Elisabeth Snead: Small Animal Clinical Sciences, Western College of Veterinary Medicine (WCVM); liz.snead@usask.ca 306-966-7068

Dr. Chantal McMillan: Department of Clinical and Diagnostic Sciences, University of Calgary Faculty of Veterinary Medicine, +1 (403) 210-6002

Funding Source(s):

Companion Animal Health Fund, Western College of Veterinary Medicine

Purpose and Objective of the Study:

Similar to humans, there has been a major increase in the incidence of diabetes in pet cats, which is often associated with obesity related changes in insulin sensitivity. Management of these cats could be greatly improved if we could more accurately predict and monitor how individual cats will respond to treatment, or even identify a 'pre-diabetic state,' in which cats have some degree of insulin resistance but are not yet clinically diabetic. At this point in time, there are no tests available to accomplish this. Nesfatin-1 is a recently discovered hormone that plays a role in regulating food intake, blood sugar and insulin levels. Studies in humans have shown that increases in nesfatin-1 are correlated with other signs of insulin resistance in obese and diabetic people, and that nesfatin-1 levels decrease following successful treatment of type 2 diabetes.

Description of the Procedures: If your pet participates in this study, we will ask you a few questions about their medical history. Your pet will have a physical exam, and a single small blood and urine sample will also be taken to look at a few health parameters in addition to nesfatin-1 levels. The urine sample will be collected by placing a needle through the abdominal wall into the urinary bladder (cystocentesis) using ultrasound guidance. The method of blood and urine collection will be performed according to routine VMC guidelines and are standard procedures for collecting blood and urine samples in small animal patients. However, we will be collecting a slightly larger blood sample than routine for the purposes of our study. The blood and urine sampling should take about 30 minutes and will take place at the Veterinary Medical Centre.

Knowledge Transfer: The results of this study will be analyzed and reported in a manuscript with the goal of being published in a scientific journal. Results of blood work to assess general health will be communicated to you.

Potential Benefits: Diabetes mellitus is a common disease in cats that requires both financial and time commitments from owners. This study aims to provide new information that will not only help us to further understand the development of this condition, but also potentially help identify at risk individuals. In addition, by choosing to participate, your cat will receive a free physical exam by a veterinarian, as well as blood and urine work that will allow you to have greater knowledge of your cat's overall health.

Potential Risks and Discomforts: While small, there is a risk of your pet developing bruising at the site of the blood collection. Cystocentesis to collect a urine sample is a quick and simple procedure and risks will be minimized by use of ultrasound guidance to locate the bladder. All researchers are experienced at blood and urine collection and will be using appropriate techniques and materials to minimize these risks.

Financial Implications: There will be no cost to you for entering your animal(s) in this study. You will not be charged for any of the procedures performed solely for the study's purposes, including blood work and urinalysis to assess general health.

Confidentiality: While absolute confidentiality cannot be guaranteed, every effort will be made to ensure that the information collected for this study is kept entirely confidential. Each cat will

be deidentified with an alphanumeric code for the purpose of the study. Your name or that of your animal(s) will not be attached to any information nor mentioned in any study report, nor be made available to anyone except the research team. Results of this study are intended for publication in scientific journals and presentation at related conferences and workshops, but your identity or that of your animal(s) will not be revealed.

Data Storage: All research materials will be stored in a secured location under lock- and-key for a period of 5 years post publication minimum as per the U of S Responsible Conduct of Research Policy, see: http://www.usask.ca/university_secretary/policies/research/8_25.php).

Voluntary Participation: Your animal's participation is voluntary, and you may withdraw your animal(s) from the research project for any reason, at any time, without penalty of any sort. If you do not wish to participate, you do not have to provide any reason for your decision, nor will you lose the benefit of any veterinary care your animal(s) is receiving. If you withdraw your animal(s) from the research project any data collected about him or her during their enrollment in the study will be retained for analysis.

Questions: If you have any questions concerning the research project, please feel free to ask at any point; you can contact the primary investigators (Dr. Meachem or Dr. Toh) by phone or e-mail using the contact details listed above. This research project has been approved on ethical grounds by the University of Saskatchewan Animal Research Ethics Board. Any questions regarding the ethical conduct of this research may be addressed to that committee through the Research Ethics Office (306-966- 7928). Participants from outside of Saskatoon may call toll free 1-888-966-2975.

Consent to Participate: Please read and initial the following statements to signify your agreement and sign below.

_____ I have read or have had the consent form read to me and I understand the consent form.

_____ I have had an opportunity to ask questions and my/our questions have been answered.

_____ I freely consent to entering my animal(s) in this study.

_____ I have been told that a signed and dated copy of this Consent Form will be given to me for my records.

_____ I am at least 18 years of age and am the legal owner of the animal(s) or am authorized to make decisions regarding this(these) animal(s) on the owner's behalf.

_____ Signature of Owner or Agent

_____ Date

_____ Signature of Individual conducting the Consent Process

_____ Date

Consent Form For Animal Owners – Nesfatin-1 Tissue Study

You have been invited to enter _____ (pet's name) in a research project entitled **"Evaluation of nesfatin-1 in lean, obese and diabetic cats"**. Please read this form carefully, and ask questions you might have.

Researcher(s):

Dr. Melissa Meachem: Department of Veterinary Pathology, Western College of Veterinary Medicine (WCVM). Primary Investigator; melissa.meachem@usask.ca, 306-966-2688
Dr. Peter Toh: Graduate Student, Department of Veterinary Pathology, Western College of Veterinary Medicine. Co-Primary Investigator; peter.toh@usask.ca, 306-966-7309
Dr. Elisabeth Snead: Small Animal Clinical Sciences, Western College of Veterinary Medicine (WCVM); liz.snead@usask.ca 306-966-7068
Dr. Chantal McMillan: Department of Clinical and Diagnostic Sciences, University of Calgary Faculty of Veterinary Medicine, +1 (403) 210-6002

Funding Source(s): Companion Animal Health Fund, Western College of Veterinary Medicine

Purpose and Objective of the Study:

Similar to humans, there has been a major increase in the incidence of diabetes in pet cats, which is often associated with obesity related changes in insulin sensitivity. Nesfatin-1 is a recently discovered hormone that plays a role in regulating food intake, blood sugar and insulin levels. Studies in humans have shown that increases in nesfatin-1 are correlated with other signs of insulin resistance in obese and diabetic people, and we are investigating if similar changes occur in cats. Little is currently known about the tissue expression of this new hormone throughout the gastrointestinal system, and how that may change with obesity and diabetes in cats. Investigating how the expression of nesfatin-1 changes may help us understand how diabetes develops in cats, and assist in the development new ways of monitoring and/or predicting cats that are at risk of developing diabetes.

Description of the Procedures: Restraint and humane euthanasia will be performed by trained personel according to hospital guidelines, via the placement of an intravenous catheter and

administration of pentobarbital sodium. Gentle handling techniques will be used at all time to minimize stress and discomfort. A blood sample will be collected if recent blood work has not been performed, this will be collected via the catheter already placed for euthanasia. After euthanasia has been confirmed, small pieces of tissue (stomach, pancreas, skeletal muscle and abdominal fat) will be collected via an abdominal incision using surgical instruments. After this has been completed your cat will be released back to you for burial/ cremation as previously arranged with the attending veterinarian.

Knowledge Transfer: The results of this study will be analyzed and reported in a manuscript with the goal of being published in a scientific journal.

Potential Benefits: Diabetes mellitus is a common disease in cats that requires both financial and time commitments from owners. This study aims to provide new information that will help us to further understand the development of this condition.

Potential Risks and Discomforts: Slight discomfort may be felt during placement of the intravenous catheter, and sedation may be administered if any distress/ discomfort is apparent.

Financial Implications: There will be no cost to you for entering your animal(s) in this study.

Confidentiality: While absolute confidentiality cannot be guaranteed, every effort will be made to ensure that the information collected for this study is kept entirely confidential. Each cat will be deidentified with an alphanumeric code for the purpose of the study. Your name or that of your animal(s) will not be attached to any information nor mentioned in any study report, nor be made available to anyone except the research team. Results of this study are intended for publication in scientific journals and presentation at related conferences and workshops, but your identity or that of your animal(s) will not be revealed.

Data Storage: All research materials will be stored in a **secured location under lock- and-key for a period of 5 years post publication minimum as per the U of S Responsible Conduct of Research Policy, see:** http://www.usask.ca/university_secretary/policies/research/8_25.php).

Voluntary Participation: Your animal's participation is voluntary, and you may withdraw your animal(s) from the research project for any reason, at any time, without penalty of any sort.

Questions: If you have any questions concerning the research project, please feel free to ask at any point; you can contact the primary investigators by phone or e-mail using the contact details listed above. This research project has been approved on ethical grounds by the University of Saskatchewan Animal Research Ethics Board. Any questions regarding the ethical conduct of this research may be addressed to that committee through the Research Ethics Office (306-966-7928). Participants from outside of Saskatoon may call toll free 1-888-966-2975.

Consent to Participate: Please read and initial the following statements to signify your agreement and sign below.

_____ I have read or have had the consent form read to me and I understand the consent form.

_____ I have had an opportunity to ask questions and my/our questions have been answered.

_____ I freely consent to entering my animal(s) in this study.

_____ I have been told a signed and dated copy of this Consent Form will be given to me for my records.

_____ I am at least 18 years of age and am the legal owner of the animal(s) or am authorized to make decisions regarding this(these) animal(s) on the owner's behalf.

_____ Signature of Owner or Agent

_____ Date

_____ Signature of Individual conducting the Consent Process

_____ Date

Appendix C: Study Protocols

RNEasy Tissue Prepration & RNA Extraction Protocol – Stomach and Pancreas

Materials Needed Before Starting (all labelled)

- 2x RINO RNA Lysis Tubes
- 2x RNEasy Columns
- 2x 1.7ml Eppendorf Tubes
- 2x 2ml Tubes (Supplied with kit)
- 2x 1.5ml Tubes (Supplied with kit)
- 2x 1.7ml Eppendorf tubes (empty, for balancing in centrifuge)

Perform extractions on 2 tissues at a time

Initial Tissue Preparation

- Place three petri dishes in -20 freezer for at least 10 minutes to cool down
- Take tissue out of -80 **one at a time**
- **For Each tissue**
    - Cut a small slice of tissue off, and put it in one of the -20 petri dishes
        - Put tissue straight back in the -80 Freezer
    - Repeat for the other tissue
    - Cut small pieces of tissue off the original slice
        - **For Pancreas and Stomach:** Cut off 10-20mg tissue with **300uL** buffer
- Place tissue in **RINO RNA Lysis Tube** with **300ul** Buffer RLT + ß-ME
    - **Put both tubes into bullet blender for the following times**
        - **Stomach and Pancreas –** 4 minutes at speed 8

RNA Extraction

- Spin RINO RNA Lysis tube again in regular centrifuge, **3 minutes at max speed**
- Add equivalent volume of **70% Ethanol** (350ul), mix by pipetting
- Extract supernatant, place it into new 1.7ml Eppendorf
    - **For pancreas and stomach:** Put 250uL of sample in tube with ethanol
        - Add extra 100uL of buffer RLT
- Transfer up to 700ul (i.e. the whole sample) into the **RNEasy spin column**
    - Centrifuge 15-30s at >10,000rpm/ 8000g – **Discard flow-through**
- Add **700ul Buffer RW1** to spin column
    - Centrifuge 15-30s >10,000rpm – **Discard flow through**
- Add **500ul Buffer RPE** to column
    - Centrifuge 15-30s >10,000rpm – **Discard flow through**
- Add **500ul Buffer RPE** to column
    - Centrifuge **2 minutes** >10,000 – **Place column into supplied new 2ml tube**
- Centrifuge column **1 minute @ full speed**
- Place column in a supplied 1.5ml column
    - **For All Tissues: Add 50uL RNAse free water**
- Centrifuge Column for 1 minute at >10,000rpm/ full speed

Measure concentration/ absorbance ratio on Nanodrop

- Zero machine with water twice, then add sample
    - Re-Zero with Water between analysis
    - Check concentration, 260/230 and 260/280 ratio
- Then dilute RNA to 50ng/ul (as using 2ul of sample in each well)
    - Make 2 dilutions, each 100ul
    - DNAse treat one sample and freeze in 50uL aliquots
    - Re-check DNAse treated sample on Nanodrop
    - Freeze one 100ul sample untreated as a backup
        - And freeze remainder of undiluted sample (if still some leftover)

<u>TRIzol RNA Extraction Protocol</u> – For Muscle and Fat

Tubes Required for each set of 2 tissues

- 2 x RINO RNA Lysis Tubes
- 6 x 1.7ml Eppendorf Tubes
- 2 x spin columns 2 x extra collection tubes 2 x recovery tubes

Tissue Extraction

- Put 600ul of Trizol reagent into a labelled RINO tube
- **Fat:** 2-3 thin slices of tissue cut in the -20 freezer
- **Muscle:** 1-2 thin slices of tissue cut in the -20 freezer
- Add tissue into RINO tube with TRIzol
- Homogenize Tissue: Bullet Blender Setting – **Speed 10 for 4 Minutes**

RNA Processing

1. Place 600ul of the homogenized samples into a new Eppendorf tube
    a. **Must take it out of the RINO tube before centrifuging**
2. Centrifuge sample for 5 minutes at max speed
3. Transfer 400ul of the supernatant (avoid fat/tissue sediment) into new Eppendorf tube
4. Incubate for 5 minutes at room temperature
5. Add 80uL of chloroform (or 20ul of chloroform per 100ul of supernatant), mix gently then incubate for another 2-3 minutes
6. Centrifuge sample for 15 minutes at max speed
7. Transfer 200-250uL of sample into a new Eppendorf tube
    a. **Make sure you only take the top layer and don't touch the interphase layer**
8. Add 200-250ul of ethanol and vortex to mix

Binding the RNA to the Membrane and Washing the Membrane

9. **Transfer Trizol/ Ethanol sample into a spin cartridge**
10. Centrifuge at max speed for 20 seconds, discard flow through

11. **Add 700ul of wash buffer I**
12. Centrifuge max speed for 20 seconds, discard flow through
13. **Put spin column into a new collection tube**
14. **Add 500ul wash buffer II**
15. Centrifuge max speed for 20 seconds, discard flow through
16. **Add 500ul wash buffer II**
17. Centrifuge max speed for 20 seconds, discard flow through

Eluting the RNA

18. Centrifuge at max speed for 1 minute to dry the membrane
19. **Insert spin cartridge into a collection tube**
20. Add 100ul RNA free water **to the centre of the spin cartridge**
21. Incubate for 1 minute
22. Centrifuge at max speed for 2 minutes

Measure concentration/ absorbance ratio on Nanodrop

- Zero machine with water twice, then add sample
    - Re-Zero with Water between analysis
    - Check concentration, 260/230 and 260/280 ratio
- Then dilute RNA to 50ng/ul (as using 2ul of sample in each well)
    - Make 2 dilutions, each 100ul
    - DNAse treat one sample and freeze in 50uL aliquots
    - Re-check DNAse treated sample on Nanodrop
    - Freeze one 100ul sample untreated as a backup
        - And freeze remainder of undiluted sample (if still some leftover)

DNAse Digestion Protocol (After initial RNA extraction and nanodrop measurement)

Turn on water bath and set at 37C

- DNAse I Buffer and DNAse Inactivation Reagent need to be defrosted
- rDNAse I does not need defrosting

For 50uL of Sample (<200ng/uL RNA)

1. Add 5uL DNAse I Buffer (0.1 volume) + 1uL rDNAse I and mix gently (e.g. touch on the vortex)
2. Incubate at 37C for 20-30 minutes
3. Add 5uL resuspended DNAse Inactivation Reagent (0.1 volume)
    a. Vortex sample before use
4. Incubate at room temperature for 2 minutes
    a. Vortex 2-3 times during this time to ensure proper mixing
5. Centrifuge >12,500rpm for 1.5 minutes
    a. Place supernatant in new tube

For 100uL of Sample (<200ng/uL RNA)

1. Add 10uL DNAse I Buffer (0.1 volume) + 1uL rDNAse I and mix gently (e.g. touch on the vortex)
2. Incubate at 37C for 20-30 minutes
3. Add 10uL resuspended DNAse Inactivation Reagent (0.1 volume)
    a. Vortex sample before use
4. Incubate at room temperature for 2 minutes
    a. Vortex 2-3 times during this time to ensure proper mixing
5. Centrifuge >12,500rpm for 1.5 minutes
    a. Place supernatant in new tube

Note: DNAse Inactivation Reagent can become fluid depleted and difficult to pipette with multiple uses. If it dries out add approx. 20-25% bed volume of the remaining reagent

- E.g. approx 300uL reagent, add 60uL water

Gel Electrophoresis – Protocol

For Small Gel (8 wells)

- Get gel mold and casting tray, use balance to ensure a flat surface
    - Firmly fix mold into the casting tray
- Measure out **50ml TBE buffer** and into conical flask
- Measure out **0.5-0.55g agarose** and place into flask
- Place paper towel on microwave and put flask on top
- Microwave for **2 x 30 second bursts**, swirling/ mixing in between
    - And then an extra 10-15 seconds
    - Mixture should be completely clear
- Allow to cool slightly for 5-10 minutes
- Add **2ul** ethidium bromide and swirl to mix
- Pour into prepared mold/ casting tray
- Place comb into gel and put aside to set

For Medium Gel (20 wells)

- Get gel mold and casting tray, use balance to ensure a flat surface
    - Firmly fix mold into the casting tray
- Measure out **100ml TBE buffer** and into conical flask
- Measure out **1-1.05g agarose** and place into flask
- Place paper towel on microwave and put flask on top
- Microwave for **3 x 30 second bursts**, swirling/ mixing in between
    - And then an extra 10-15 seconds
    - Mixture should be completely clear
- Allow to cool slightly for 5-10 minutes
- Add **4ul** ethidium bromide and swirl to mix
- Pour into prepared mold/ casting tray
- Place comb into gel and put aside to set

Running Gel

- Wells should be close to the negative terminal (product runs to positive terminal)
- Place gel into station – If needed add TBE buffer to ensure gel is covered

- Place DNA ladder into well
    - **If running extracted RNA –** 10ul
    - **If running PCR product –** 8ul
- Fill wells with each product – mix samples on wax paper before loading
    - **If running extracted RNA –** 8ul sample + 2ul loading dye
        - **Run at 55V for 70-80 minutes**
    - **If running PCR product –** 5ul sample + 2ul loading dye
        - **Run at 110V for 40 minutes**

<u>One-Step PCR Protocol</u>

<u>For All Tissues</u>

Ingredients for 1 reaction

- **12.5ul Master Mix**
- **2ul F Primer (1:10 dilution)**
- **2ul R Primer (1:10 dilution)**
- **0.25ml RT mix**
- **2ul Template/ water (or enough to make up 100ng RNA)**
- **6.25ul RNAse free water (or less if more template required)**

Ingredients for 1 Minus-RT Control reaction

- **12.5ul Master Mix**
- **2ul F Primer (1:10 dilution)**
- **2ul R Primer (1:10 dilution)**
- **2ul Template (or enough to make up 100ng RNA)**
- **6.5ul RNAse free water (or less if more template required)**

Reactions Required Per Sample – TWO samples at a time

- **Each Primer Pair in Triplicate –** 9 Samples total
- **NTCs in triplicate –** 9 Samples
    - Assessing two samples, only need to do NTCs once
- **Minus-RT Control in triplicate** – 9 Samples
    - Need to do twice (one for each sample, assessing gDNA contamination in each)

Mix Required for NTCs + Sample 1 + Sample 2 In Triplicate

- Total of 9 Mixes for Each Primer Pair (27 reactions total) – So Mix up 10 reactions

Mix Required for -RT Controls – Sample 1 and Sample 2 in Triplicate

- Total of 6 Mixes for Each Primer Pair (18 reactions total) – So Mix up 7 reactions

Note

- UV light treat tube strips 20 minutes before filling (while preparing master mix)
- Take water for NTCs from the PCR water from the One-Step Kit (25uL at a time)
- Add 1 extra reaction amount per 10 reactions for each primer pair
    - Allowing for wastage during pipetting

All Tissues – 2 Tissues at a time

Ingredients for 10 reactions

- 125ul Master Mix
- 20ul F Primer (1:10 dilution)
- 20ul R Primer (1:10 dilution)
- 2.5ml RT mix
- 62.5ul RNAse free water (or less if more template required)
- 2ul Template/ water per well

Ingredients for 7 <u>Minus-RT Control</u> reaction

- 87.5ul Master Mix
- 14ul F Primer (1:10 dilution)
- 14ul R Primer (1:10 dilution)
- 45.5ul RNAse free water
- 2ul Template per well

Mix **Master Mix + RT Mix + Primer + Water in the PCR Room**

Add **RNA Template/ water control in the clean box outside the room**

PCR Parameters

- 10 minutes annealing @ 50C
- 5 minutes activation @ 95C

- 35 Cycles
    - 10 seconds @ 95 30 seconds @ 60 10 seconds @ 95
- Melt Curve Analysis @ 65C – 95C

Blood Collection + Plasma and Serum Preparation Protocol

Blood Collection

Materials needed

- Alcohol
- 23-gauge butterfly catheters
- 6ml Syringes
- 1ml and 3ml syringes
- 2 x 2ml EDTA blood tubes (Purple top, for plasma), labelled
- 1 x Serum Separator Tube (Yellow tube, for serum), labelled
- 1.5ml Eppendorf tubes, labelled

Collection Protocol

1. Try jugular venipuncture first, then medial saphenous venipuncture if necessary
2. Swab area with alcohol, and shave area if better visualisation is required
3. For Jugular Venipuncture
    a. Insert butterfly catheter, and draw 5-6mls of blood with a 6ml syringe
4. For Medial Saphenous venipuncture (restrain cat in lateral recumbency)
    a. Insert butterfly catheter, and using multiple 1 or 3ml syringes, draw 1-2mls of blood at a time to prevent clotting within the syringe
5. Immediately transfer 2mls of whole blood into each EDTA tube
6. Transfer remaining 1-2mls of blood, and whatever is left in the butterfly catheter into the serum separator tube
7. **For diabetic cats only**
    a. Use a drop of blood to measure blood glucose and blood ketones using a handheld glucometer and ketometer
8. Transfer plasma and serum to the fridge within 10 minutes of collection
9. Centrifuge/ separate plasma and serum within 30 minutes of collection

Plasma/ Serum Preparation Protocol

1. Make a blood smear with the EDTA whole blood
    a. Dry smear and stain with diff-quik solution

b. Diff-Quik method
    i. Using a pipette, flood the slide with fixative and leave for 30 seconds, then shake off the excess
    ii. Flood slide with diff-quik stain 1, leave for 30 seconds, shake off excess
    iii. Flood slide with diff-quik stain 2, leave for 30 seconds, shake off excess
    iv. Allow to air dry, then place a cover slip on the slide using mounting media
        1. Do this step in the fume hood
c. Blood smear is used to perform a manual Complete Blood Count

2. Fill 2 capillary tubes ¾ full with whole blood
    a. Spin capillary tubes in a micro-hematocrit centrifuge for 5 minutes
    b. Read PCV
    c. Break capillary tubes, and empty plasma onto a refractometer, and measure Total Protein
3. Spin plasma and serum in the low speed centrifuge for 7 minutes @ 1800rpm
    a. Pipette separated plasma and serum into labelled Eppendorf tubes
    b. Transfer to the -80 freezer and place in labelled freezer boxes
        i. Plasma stored for eventual use with nesfatin-1 assay
        ii. Serum submitted for routine serum biochemistry, fructosamine, and T4

<u>Urine Collection</u> – For lean cats, overweight cats, and diabetic cats at their first appointment

Materials Needed

- 6ml syringe
- 1 ½ inch x 25G needle
- Alcohol
- Urine Collection Tubes
- Ultrasound

Collection Protocol

- Lay cat in dorsal recumbency, restrain front legs and hind legs
- Wet caudal abdomen with alcohol
- Find bladder with ultrasound probe, and insert needle attached to syringe into the bladder using ultrasound guidance
- Collect approximately 3mls of urine, and place into urine collection tube
- Keep urine at room temperature, or store in the fridge is urinalysis will be delayed by more than 30 minutes

Urine Analysis Protocol

- Measure urine specific gravity using a refractometer
- Dip a urine dipstick into the urine before centrifugation and record the results
- If abnormalities are present on the dipstick (eg proteinuria, hematuria), then analyse urine sediment
- For sediment analysis
    - Centrifuge urine for 3 minutes @ 1800rpm
    - Discard supernatant
    - Place one drop of sediment on a slide and place cover slip over the top
        - Assess urine for cellular elements, bacteria, crystals etc.

Hematology/ Blood Smear Interpretation Protocol

At 10 x Objective

- **Look for any significant platelet clumps at the feathered edge**
- **Leukocyte Count:**
    - Count leukocytes in the monolayer
    - Average count in 5 fields, divide average by 4 (Leuks x 10^9/L)

100 x Objective

- **Evaluate RBC morphology**
    - Size, shape, colour, inclusions, arrangement/ rouleaux etc
    - E.g. spherocytes, ghosts, Heinz bodies, eccentro, kerato, echino, schizo, acantho
    - Polychromasia, macrocytes, microcytes
    - Nucleated reds

- **100 leukocyte differential count**
    - Multiply the percentages of different leukocytes by the total leukocyte count, to get the total number of each leukocyte
    - Note any significant changes in leukocyte morphology

- **Estimate Platelet Numbers**
    - Count platelet numbers in at least 5 fields and average
        - Multiply by 20 x 10^9/L
    - >10/ 100x field usually means adequate numbers
    - Look at platelet morphology, giant platelets etc.

FreeStyle Libre® Blood Glucose Monitoring Device

Standard Operating Procedure For Use In Feline Diabetics

Materials Needed

- Clippers and alcohol
- Freestyle Libre® Reader
- Freestyle Libre® Sensor
- Tissue Glue
- Tegaderm® Adhesive bandage
- Soft esophageal collar

1. Shave an area approximately 8cm x 8cm over the dorso-lateral neck
2. Clean area thoroughly with alcohol, and allow to dry
3. Apply Tissue glue around the perimeter of the sensor for additional attachment strength
4. Insert the lancet of the sensor through the skin using the provided applicator and hold applicator down for 10 seconds. This allows the adhesive to properly attach the sensor to the skin.
5. Cover the sensor Tegaderm® adhesive bandage for additional protection
6. Fit the cat with a soft collar (oesophagostomy collar or similar) to prevent help self-removal of the sensor
7. Hold the electronic reader against the device and select 'start new sensor'. This ensures that the reader will start to read results from the sensor on the cat. Wait for 1 hour as the reader warms up.
8. Take a glucose measurement 1 hour after the reader has been linked to the sensor, to ensure that the sensor is operational
9. Clients will be instructed to scan the sensor with the reader at least every 8 hours, though preferably at least 4-5 times per day.
10. Sensor and reader will be returned to the clinic once it is no longer attached. Data will be collected and saved from this period of time