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ADVANCEMENTS IN DIAGNOSTIC, THERAPEUTIC, AND MONITORING OPTIONS FOR CANINE AND FELINE DIABETES MELLITUS, CANINE HYPOADRENOCORTICISM, AND CANINE HYPERCORTISOLISM

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Abstract

Abstract

This thesis presents advancements in diagnostic, therapeutic, and monitoring approaches for canine and feline endocrinopathies, focusing specifically on diabetes mellitus (DM), hypoadrenocorticism (HA), and hypercortisolism (HC). The thesis begins with a comprehensive review of canine and feline diabetes mellitus, and canine hypoadrenocorticism and hypercortisolism. It then examines the role of nutrition in glycemic control, presenting a prospective randomized crossover study aimed at evaluating the effects of a homemade diet versus a commercial diet on glycemic control and glycemic variability of diabetic dogs. Results showed that both diets were effective options for diabetic dogs; however, the homemade diet formulated for this study demonstrated a superior glucose-lowering effect, highlighting the importance of considering this dietary approach in managing canine DM. This thesis explores the use of novel insulin analogs in the management of DM in dogs, describing the use of insulin glargine 300 U/mL and insulin degludec 100 U/mL as once-daily basal insulin in client-owned diabetic dogs, with dose titration guided by continuous glucose monitoring system (CGMS). These insulin analogs demonstrated promising outcomes with once-daily dosing, decoupling insulin injections from feeding and providing a practical alternative to traditional protocols that require 12-hourly injections of intermediate-acting insulin alongside consistent meal schedules. Hypoglycemia is a primary limiting factor in managing DM in patients receiving insulin therapy, and fear of hypoglycemia is one of the most significant factors negatively impacting the quality of life for owners of diabetic pets. Transmucosal glucagon formulations, currently used in human diabetic care, could reduce the fear of hypoglycemia of diabetic pet owners, potentially improving their quality of life. This thesis investigates the use of Baqsimi, an intranasal glucagon powder recently approved for use in diabetic people, in healthy cats. Baqsimi rapidly increased blood glucose concentrations when administered both intranasally and rectally, and was well tolerated by the cats. This study opens new insights for further investigations in diabetic cats, as this medication holds the potential to become a lifesaving treatment during hypoglycemic events. The use of CGMS rapresent a paradigm shift in veterinary diabetic management. These devices allows real-time and comprehensive assessment of interstitial glucose excursions occurring throughout the day and night, as well as of glucose variations over consecutive days, enabling clinicians to make quicker and more informed decisions about insulin dose titration. The FreeStyle Libre is currently the most studied CGMS in veterinary patients. The third generation of the device (FreeStyle Libre 3) includes several improvements over previous models, such as a sensor size reduction of approximately 60%. This thesis investigates the accuracy of the FreeStyle Libre in both diabetic and healthy cats, demonstrating good clinical accuracy across a wide glycemic range and suggesting improved tolerability of this device in cats. Although the FreeStyle Libre is increasingly used in diabetic veterinary patients, its integration into routine clinical practice remains limited due to the absence of standardized guidelines for data interpretation. To address this knowledge gap, this thesis evaluates the utility of various metrics readily available through the FreeStyle Libre in diabetic dogs. The findings indicate that FreeStyle Libre-derived metrics hold potential for assessing glycemic control in diabetic dogs, as they provide valuable insights into glucose trends that may complement traditional clinical assessments and facilitate more precise insulin management. This preliminary study is the first to assess CGMS-metrics in diabetic dogs, underscoring the necessity for further investigations to establish guidelines for CGMS data interpretation. In veterinary medicine, it is widely acknowledged that owner compliance is crucial for the successful management of DM. Consequently, this thesis examines the impact of the FreeStyle Libre on the quality of life of diabetic pet owners. The latter perceived the FreeStyle Libre as user-friendly and less stressful compared to traditional blood glucose monitoring methods, while also facilitating improved glycemic control. However, the long-term costs associated with its use were deemed challenging to afford. The limited sensor lifespan is arguably the primary drawback of FreeStyle Libre devices, which have a functional life of up to 15 days. This thesis evaluates the use of Eversense XL, a novel CGMS with a functional lifespan of up to 180 days, in diabetic dogs. This device might be considered a future alternative for glucose monitoring and could enhance adherence

and long-term use of CGMS in diabetic dogs. However, the application of the current model of the device in veterinary medicine may be limited due to excessive sensor movement, the need for daily calibrations, and high costs.

Hypoadrenocorticism is a rare endocrinopathy in dogs and up to 30% of cases have normal electrolyte concentrations at diagnosis. This form of the disease is defined as eunatremic, eukalemic hypoadrenocorticism (EEH) and might go undetected for a long period because of vague clinical signs and the absence of typical biochemical abnormalities. Consequently, EEH might be mistaken for other diseases, such as chronic gastrointestinal disease (CGD). Additionally, previous administration of glucocorticoids, frequently used in dogs with CGD, can lead to false positive results on the adrenal function tests, potentially resulting in a misdiagnosis of EEH. This thesis first presents the results of a multicenter prospective study aimed to determine the prevalence of EEH in dogs with signs of CGD, and to identify clinical and clinicopathological features for EEH and previous glucocorticoid administration. Then, it presents a single-center prospective observational study aimed at determining the timeline for recovery of the hypothalamic-pituitary-adrenal (HPA) axis in a group of 20 ill dogs treated with intermediate-acting glucocorticoids. The findings indicate that the prevalence of EEH is below 1% in dogs presenting with signs of CGD, highlighting the importance of excluding prior glucocorticoid administration to prevent misdiagnosis of EEH. However, the optimal time to test for HPA axis recovery after glucocorticoid use remains controversial due to variability of data regarding the recovery timelines. Measurement of a basal cortisol concentration is commonly used to rule out hypoadrenocorticism. However, due to the low specificity of this test, urinary corticoid-to-creatinine ratio (UCCR) has been proposed as alternative screening test for HA in dogs. This thesis present two studies aimed at investigate the performance of UCCR in diagnosing HA, before and after the change in antibody used for cortisol measurement in the Immulite 2000 chemiluminescent assay. The results indicate that urinary cortisol and UCCR are effective alternatives to basal serum cortisol for initial HA screening in dogs.

The change of Immulite 2000 antibody has introduced an average bias of -23% in canine serum and -70% in urine cortisol measurements. Consequently, previously established diagnostic cut-offs for canine hyperadrenocorticism require re-evaluation. This thesis presents preliminary results from two studies aimed at establishing new reference intervals and evaluating the diagnostic performance of UCCR and the low-dose dexamethasone suppression test (LDDST) using the updated cortisol antibody. The sensitivity of UCCR for diagnosing HA was lower than previously reported, and the optimal cut-off for LDDST 8-hour cortisol was found to be >1.2 µg/dL, lower than the currently accepted threshold of >1.4 µg/dL. Naturally occurring hypercortisolism can disrupt calcium and phosphate homeostasis through various mechanisms. This condition, previously referred to as “adrenal secondary hyperparathyroidism”, may resolve in dogs undergoing medical treatment with trilostane. However, the combined effects of medical and nutritional interventions on calcium-phosphate homeostasis in dogs with HC have not yet been explored. The final study in this thesis examines the effect of a therapeutic commercial diet formulated for the management of calcium oxalate (CaOx) urolithiasis on calcium and phosphate homeostasis in dogs with HC treated with trilostane. Preliminary findings from this study suggest that the use of a CaOx-specific therapeutic diet may help restore calcium and phosphate homeostasis in dogs with HC undergoing treatment with trilostane. In conclusion, the overall results of this thesis provide relevant insights into various endocrine diseases, aiding clinicians in improving the diagnosis and daily management of veterinary patients affected by diabetes mellitus, hypoadrenocorticism, and hypercortisolism.

Chapter 1 – Aims and scope of the thesis

The introduction section of this thesis (**Chapter 2**) presents a comprehensive review of diagnostic, therapeutic, and monitoring approaches for diabetes mellitus (DM), hypoadrenocorticism (HA), and hypercortisolism (HC) in veterinary medicine. Particular emphasis is placed on the use of novel insulin analogs in diabetic dogs and continuous glucose monitoring system (CGMS) in diabetic veterinary patients. Additionally, diagnostic approaches for canine hypoadrenocorticism and hypercortisolism are discussed, focusing on the risk of misdiagnosis and the performance of methods used for serum and urinary cortisol measurement.

Diabetes mellitus is a heterogeneous group of diseases with multiple etiologies characterized by hyperglycemia resulting from inadequate insulin secretion, inadequate insulin action or both.[1] It is one of the most common canine and feline endocrinopathies, affecting approximately 1 in 300 dogs and 1 in 200 cats.[2-4] Diet plays a key role for the management of dogs and cats with DM.[5] In dogs, most studies assessing the role of nutrition in the glycemic control have focused on the effects of dietary fiber and carbohydrates (CHO).[6-8] However, there is currently no consensus on recommended types and levels of dietary fiber and CHO in diabetic pet foods. The choice of diet ultimately depends on the weight of the diabetic dog, concurrent diseases, and both owner and dog preferences. Although the majority of pet owners prefer commercial diets (CD), some are interested in providing a homemade diet (HMD) for their animals. Homemade diets could prove beneficial for dogs with DM, as their nutritional content can be customized to meet the individual patient's needs, and clinical trials evaluating its use in client-owned diabetic dogs are warranted. **Chapter 3.1** aims to evaluate the effects of a CD and a HMD on glycemic control and glycemic variability of client-owned dogs with stabilized DM, monitored with a continuous glucose monitoring system (CGMS).

Insulin treatment is the cornerstone of DM management in dogs. Ideally, insulin treatment in dogs should mimic the physiology of endogenous insulin secretion, which is characterized by a “basal-bolus” pattern.[9] However, to minimize costs and the need for multiple daily injections, insulin treatment in dogs traditionally has relied on the use of intermediate-acting insulin suspensions administered at the time of feeding.[10] These formulations however are associated with some drawbacks such as the need to match insulin administration to consistent feeding, marked day-to-day variability, and increased risk of hypoglycemia.[11-15] Diabetology in humans has shifted to using recombinant insulin analogs which are designed to closely mimic physiologic insulin secretion and to have minimal within-day and between-day variability, which are important features in minimizing hypoglycemic events.[16-17] In dogs, insulin glargine 300 U/mL (IGla300) and insulin degludec 100 U/mL (IDeg) have long duration of action, peakless time-action profile, and low potency, making them suitable for use as a basal insulin.[15,18] **Chapter 3.2** and **3.3** describe two treatment protocol using IGla300 or IDeg as basal once-daily insulins in client-owned diabetic dogs with CGMS monitoring used for dose titration.

Hypoglycemia is a major limiting factor in the management of DM in patients receiving insulin therapy. In surveys that investigated the quality of life of diabetic pet owners, as well as perceived quality of life of their diabetic pets, owners’ fears of hypoglycemia had one of the largest negative impacts on their quality of life.[19,20] The American Diabetes Association recommends that glucagon is routinely prescribed to people who are at risk for severe hypoglycemic episodes.[21] Glucagon is not routinely prescribed to diabetic dogs and cats receiving insulin therapy, and its use in veterinary medicine is limited to the treatment of hypoglycemia refractory to intravenous dextrose administration. Baqsimi is an intranasal glucagon powder medication recently approved for use in diabetic people for severe hypoglycemic events, which is formulated to be passively absorbed through the nasal mucosa.[23] As a transmucosal glucagon formulation, Baqsimi has the potential to be used emergently at home by owners to treat life-threatening hypoglycemia and with no need for technical expertise. **Chapter 3.4** aims to evaluate if Baqsimi, administered

intranasally and rectally, is effective in raising blood glucose concentrations in healthy cats and to describe acute adverse reactions to its administration.

Insulin treatment necessitates close monitoring in cats and dogs with DM.[23,24] In the last decade, CGMS have revolutionized the management of DM in both human and veterinary medicine.[25,26] These devices measure interstitial glucose concentrations (IG) on a minute-by-minute basis over consecutive days or weeks, reducing blood sampling-associated patient discomfort and greatly increasing information on glucose fluctuations and trends.[25-27] The FreeStyle Libre (Abbott Laboratories) is currently the most studied CGMS in veterinary patients. The accuracy of the first (FSL1) and the second generation (FSL2) has been previously evaluated in veterinary diabetic patients.[28-30] In 2020, a third generation of the device, FreeStyle Libre 3 (FSL3), has been licensed for use in diabetic people. The FSL3 measures IG using the same sensing technology as the FSL2. However, the FSL3 features a newly designed one-piece applicator, lasts longer (up to 15 days) and the sensor is approximately 70% smaller than previous FSL models.[31] A recent study demonstrated that the FSL3 provides accurate glucose measurements across a broad glycemic range in diabetic people.[31] The smaller size of the FSL3 could provide significant advantages for veterinary patients, particularly in cats. The **Chapter 3.5** and **3.6** assess the analytical and clinical accuracy of FSL3 in diabetic and healthy cats, respectively.

The FreeStyle Libre transfers IG data from the sensor to the FreeStyle LibreLink mobile application, and the data is automatically uploaded to the LibreView system.[26] Libreview is a cloud-based diabetes management system that generates comprehensive glucose reports, providing both visual and statistical summaries of glucose metrics. These metrics include mean glucose (MG) and the percentages of time below range (TBR%), time in range (TIR%), and time above range (TAR%), as well as glycemic variability expressed as the percent coefficient of variation (CV%).[26] In human medicine, an international panel of CGMS experts recently developed consensus guidelines to provide clinicians, researchers, and individuals with DM with standardized recommendations for using, interpreting, and reporting CGMS-derived metrics in routine clinical care and research.[32,33] Although the FreeStyle Libre is increasingly used in veterinary diabetic patients, its integration into routine clinical practice remains limited due to the absence of standardized guidelines for data interpretation. **Chapter 3.7** aims to evaluate the utility of various CGMS-derived metrics readily available through the FreeStyle Libre in diabetic dogs.

In veterinary medicine, it is generally accepted that owner compliance is essential for successfully treating DM.[34] The disease and the treatment commitments are likely to have a considerable impact on owners' daily routines and quality of life (QoL) and might represent a significant temporal, financial, and emotional burden. Therefore, it is essential to consider the effects of diabetes management and various monitoring methods on the QoL of diabetic pet owners. **Chapter 3.8** explores the impact of FreeStyle Libre on the QoL of diabetic pet owners and their satisfaction with its usability.

Despite their good clinical accuracy, the limited sensor lifespan is a drawback of the FreeStyle Libre devices, with a functional life of up to 15 days. A novel CGMS (Eversense XL, Senseonics) equipped with a long-term sensor has recently been developed for humans with DM.[35] The main advantages of Eversense XL are the extended life of up to 180 days, the reduced need for sensor replacement, and the flexibility of being able to remove the external transmitter.[35] However, unlike the transcutaneous CGMSs, the long-term sensor has to be implanted and removed from the skin by means of a minimally invasive surgical procedure. Recent investigations have shown that Eversense is safe and accurate for use in diabetic people.[36,37] **Chapter 3.9** describes the clinical use of Eversense XL in three diabetic dogs, and the correlation between IG measured by Eversense XL, IG measured by FreeStyle Libre and blood glucose measured by a portable-blood glucose meter previously validated for use in dogs.

Hypoadrenocorticism is a rare endocrinopathy in dogs.[38] Primary HA refers to bilateral adrenal gland destruction and accounts for more than 95% of cases.[39] Secondary HA, a much rarer condition, is because of reduced ACTH secretion from the pituitary gland.[39] Up to 30% of dogs with primary HA have normal electrolyte concentrations at diagnosis.[40-42] This form of the disease is therefore defined as eunatremic, eukalemic hypoadrenocorticism (EEH), previously referred to as "atypical' HA".[43-45] Eunatremic, eukalemic hypoadrenocorticism might go undetected for a long period because of vague clinical signs and the absence of typical biochemical abnormalities. Consequently, EEH might be mistaken for other diseases, such as chronic gastrointestinal disease (CGD). Diagnosing HA depends on adrenal gland function testing, such as the adrenocorticotropic hormone stimulation test (ACTHst). However, previous administration of glucocorticoids, frequently used in dogs with CGD, can lead to false positive results on the ACTHst, potentially resulting in a misdiagnosis of EEH. The study presented in **Chapter 4.1** aims to determine the prevalence of EEH in dogs with signs of CGD and to identify the clinical and clinicopathological features which might help in differentiating dogs with EEH from those with CGD, and to recognize previous glucocorticoid administration in dogs with CGD. Currently, no guidelines exist regarding the required time until the ACTHst can be carried out after a dog has been treated with different glucocorticoid formulations. Generally, the extent and duration of suppression of the hypothalamic-pituitary-adrenal (HPA) axis depends on the dose, potency, half-life, and duration of glucocorticoid treatment.[46] Few published studies are available regarding the duration of HPA axis suppression in dogs receiving systemic GCs.[47-53] However, the majority of these studies were carried out on healthy experimental dogs and, as such, possible interference on HPA axis from concurrent diseases has not been investigated. Moreover, in clinical practice, gradual tapering of the glucocorticoid dose is recommended if the treatment lasts more than 2 weeks, or if high doses are used.[46] **Chapter 4.2** aims to assess the timeline for recovery of the HPA axis in a group of ill dogs treated with systemic intermediate-acting glucocorticoids.

Basal serum cortisol concentration is commonly used to rule out HA. Using a cut-off value of ≥2 µg/dL (≥55 nmol/L), the negative predictive value is reported to be between 99.8 and 100%.[54-56] However, because of the low specificity of the test (20%-78.2%), up to 33% of dogs with CGD, but without HA, have a BSC <2 µg/dL (<55 nmol/L).[54-57] Therefore, due to the low specificity of the test, an ACTHst must be performed in dogs with BSC ≤2 µg/dL (≤ 55 nmol/L) to exclude HA, resulting in increased diagnostic costs and time for the client. The urinary corticoid-to-creatinine ratio (UCCR) provides an integrated measurement of corticoid production over a given interval, thereby overcoming the problem of fluctuations in plasma concentrations.[58] The main advantages of UCCR measurement are that it requires only a single urine sample, is easy to perform, and is relatively cost-effective. The UCCR is commonly used as a screening test for dogs with spontaneous HC.[59] However, the use of this diagnostic test has been infrequently evaluated in diagnosing spontaneous HA. In **Chapter 4.3** the diagnostic performances of UCCR are evaluated in dogs with HA. In veterinary patients, serum and urinary cortisol concentrations are commonly measured using a validated chemiluminescent immunoassay (Immulite 2000 cortisol). Unfortunately, recently there was a change in the Immulite 2000 antibody used for cortisol measurement. An initial review by the European Society of Veterinary Endocrinology (ESVE)—Endocrine Quality Assurance suggested that the cortisol values measured with the new antibody were lower (average bias -70%) than the values obtained with the previous antibody.[60] Based on the above findings, the use of the new antibody might result in different diagnostic performances. Therefore, **Chapter 4.4** evaluate the reference intervals and the diagnostic performances for urinary cortisol and UCCR using the new cortisol antibody.

Naturally occurring HC or Cushing's syndrome is a common endocrine disorder in dogs.[61-62] The most common causes of naturally-occurring HC are adrenocorticotrophic hormone (ACTH)-secreting pituitary adenoma (pituitary-dependent HC) and cortisol-secreting adrenocortical tumor (adrenal-dependent HC).[63] Currently available diagnostic tests for HC have some limitations, often yielding false-positive or false-negative results, and there is no universally accepted

gold standard test. Consequently, diagnosing HC is a complex process that requires thorough evaluation of clinical signs, clinicopathological abnormalities, imaging findings, and endocrine test results. Careful selection of appropriate cases for specific endocrine tests is essential to improve diagnostic accuracy. Common diagnostic tests for investigating HC include the low-dose dexamethasone suppression test (LDDST), the ACTHst, and the UCCR.[59,63] The diagnostic performance of endocrine tests used for HC diagnosis has been previously reported.[59,63] However, a recent change in the Immulite 2000 antibody used for cortisol measurement has been associated with an average bias of -23% in canine serum and -70% in urine.[60] Consequently, the previously established cut-off points for diagnosing canine HC require re-evaluation. **Chapters 5.1** and **5.2** present new reference intervals and assess the diagnostic performance of the LDDST and UCCR using the currently available cortisol antibody.

In both dogs and people, HC has been shown to affect calcium-phosphate homeostasis.[64-68] In dogs with HC, abnormalities associated with disrupted calcium-phosphate homeostasis include hyperphosphatemia, elevated serum parathyroid hormone (PTH) concentration, decreased urinary phosphate excretion, and increased urinary calcium excretion.[65-67] Moreoveor, a recent study reported lower serum 25-(OH)-Vitamin D and plasma fibroblast growth factor-23 (FGF-23) concentrations in dogs with HC compared to controls.[68] Dogs with HC exhibit several consequences of altered calcium-phosphate homeostasis, such as calcium-containing urolithiasis, calcinosis cutis, and soft tissue mineralization.[63] It has been reported that dogs diagnosed with HC are ten times more likely to develop calcium oxalate (CaOx) uroliths than individuals of the same breed without the condition.[69] Although the pathogenesis of CaOx urolithiasis remains incompletely understood, increased urinary calcium excretion is likely a key predisposing factor.[70] In dogs with CaOx urolithiasis, due to the absence of dietary dissolution therapies, prevention is critically important and should focus on identifying and managing underlying risk factors. Prescription diets specifically formulated to reduce the formation of calcium oxalate uroliths are commercially available for this purpose. These diets are formulated with reduced calcium and protein content and are designed to lower urine specific gravity while promoting an increase in urinary pH.[70] Currently, there are no established guidelines for the nutritional management of dogs with HC, and most affected patients are typically fed standard adult maintenance diets. Therapeutic commercial diets formulated to prevent CaOx urolithiasis may have the potential to reduce urinary calcium excretion; however, their efficacy in dogs with HC has not been investigated. Therefore, **Chapter 5.3** aims to evaluate the effects of a therapeutic commercial diet formulated to prevent CaOx uroliths on calcium-phosphate homeostasis in a cohort of dogs with Cushing's syndrome.

In **Chapter 6**, the results, limitations, conclusions, and future perspectives of the studies included in this thesis are discussed.

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Chapter 2 – General introduction

CANINE AND FELINE DIABETES MELLITUS

Diabetes mellitus (DM) is a common endocrine disorder in dogs and cats. It is a treatable condition that requires excellent owner compliance and effective communication between the owner and the veterinary team.[1,2] Treatment for diabetic dogs and cats includes medical therapy, dietary management, discontinuation of diabetogenic drugs, and prevention or control of any concurrent diseases (Table 1).[1-3] Due to the various factors influencing the diabetic state and the variable response to therapy, diabetes treatment is often complex. Successful DM management is indicated by minimal or no clinical signs of diabetes (i.e., polyuria, polydipsia, polyphagia, and weight loss), avoidance of complications (e.g., hypoglycemia, diabetic ketoacidosis), and maintaining a good quality of life for both the pet and the owner. In cats, achieving diabetic remission is a reasonable goal.[3]

Regular monitoring is crucial for successfully achieving these goals. Monitoring options include clinical signs observed by the owner, blood glucose curves (BGC), glycated proteins (fructosamine and glycated hemoglobin), and continuous glucose monitoring systems (CGMS).[1,2] Continuous glucose monitoring systems are nowadays used with increasing frequency in diabetic dogs and cats, marking a significant paradigm shift in the management of diabetes in veterinary medicine. Some owners may find it challenging to comprehend the nature of diabetes and its various treatments and monitoring methods. Therefore, it is important to provide owners with detailed written information about all technical aspects of DM and offer easy access to care if needed. Furthermore, treatment and monitoring should adhere to a precise and comprehensive protocol. The prognosis for dogs and cats diagnosed with DM depends partly on owner's commitment to managing the condition, ease of glycemic control, presence and reversibility of concurrent disorders, prevention of chronic complications associated with diabetes, and minimization of treatment impact on the owner's quality of life.[1,2] In the author's experience, with appropriate owner care, regular veterinary evaluations, and effective client-veterinarian communication, diabetic dogs and cats can maintain a good quality of life over extended periods.

DISEASE	SPECIES
Obesity	Dogs, cats
Infection (e.g., urinary tract infection)	Dogs, cats
Hypothyroidism	Dogs > Cats
Hyperthyroidism	Cats
Disease of the oral cavity	Dogs, cats
Chronic inflammation (e.g., chronic enteropathy)	Dogs, cats
Hyperlipidemia	Dogs
Cushing's Syndrome	Dogs > Cats
Chronic kidney disease	Cats > Dogs
Acromegaly (Hypersomatotropism)	Cats
Chronic pancreatitis	Cats > Dogs
Diestrus in intact female	Dogs
Neoplasia	Dogs, cats

Table 1. Concurrent diseases implicated in insulin resistance in diabetic dogs and cats.

Dietary therapy

Nutritional management is an integral component of DM treatment. When properly aligned with the insulin treatment strategy, it can significantly enhance both glycemic control and the quality of life of diabetic pets. Commercial diets (CD) formulated for diabetic dogs are characterized by moderate to high fiber, high-quality protein, and restricted fat content.[1,4] Additionally, all dry diabetic CD and the majority of wet CD contain digestible "complex" carbohydrates (CHO), primarily in the form of starch.[4] The starch content in pet food can vary significantly, with higher levels typically found in dry products, where starch is essential for the formation of extruded dry kibble.[4] The dietary fiber content is advantageous for managing overweight patients and is thought to enhance glycemic control in diabetic dogs.[1] The ability of the soluble fraction of dietary fiber to form a viscous gel is critical, as it hinders the convective transfer of glucose and water to the intestinal absorptive surface, thereby slowing intestinal glucose absorption. Rapidly fermentable viscous soluble fibers (e.g., gums, pectin) impede glucose diffusion more effectively than insoluble fibers (e.g., cellulose,

hemicellulose), making them more beneficial for glycemic control.[1] However, there is currently no consensus on recommended types and levels of dietary fiber and CHO in diabetic pet foods. Moreover, diets high in fiber content, designed for weight loss, should not be fed to underweight veterinary patients.[1]

In diabetic cats, correcting obesity is the most beneficial step that can be taken to improve glycemic control. Additionally, limiting dietary carbohydrates, which can be achieved primarily through the use of canned or wet diets, may further enhance glycemic control and increase the likelihood of remission in diabetic cats.[5,6] Diabetic pets are often middle-aged to elderly, which increases the likelihood of established dietary preferences or concurrent conditions (e.g., chronic kidney disease) with very different nutritional requirements. For these animals, a standard diabetic diet may not be optimal.[5] It is also important that the owner's preferences regarding diet choice are acknowledged and incorporated into the overall treatment plan.[5] Therefore, the choice of diet ultimately depends on the weight of the diabetic pet, concurrent diseases, and both owner and animal preferences. Although the majority of pet owners prefer commercial diet, some are interested in providing a homemade diet (HMD) for their dogs and cats.[7] The preparation of HMD may enhance owners' sense of involvement with their pets, and anecdotal evidence suggests that this practice is increasing.[8] Homemade diets could prove beneficial for diabetic pet, as their nutritional content can be customized to meet the individual patient's needs.

Insulin therapy

Insulin therapy is the cornerstone of the treatment regimen in dogs and cats with DM. Insulin formulations differ in terms of their average time-action profiles, day-to-day variability, cost, method of administration, and other characteristics. Currently, two "veterinary" insulin formulations are approved for use in dogs and cats, but there are more than a dozen "human" formulations available on the market. Some of these "human" insulin formulations are routinely used in the treatment of dogs and cats (Table 2). A smart insulin choice should take into account disease pathophysiology (including concurrent diseases), insulin-related factors (such as insulin pharmacology, cost, and regional prescribing regulations), pet and owner compliance, diet (composition and frequency), monitoring strategy, and therapeutic goals.[9] Therefore, no insulin formulation should be considered "best" by default. Rather than looking for an insulin formulation that is considered "best" for a general population, it is more appropriate to seek the "smart" insulin choice, tailored to the specific clinical situation.

INSULIN	BRAND NAME	CONCENTRATION SYRINGE/PEN	DOGS		CATS	
			Starting dose	Frequency	Starting dose	Frequency
Lente	Vetsulin® Caninsulin®	40 U/mL – Syringe/pen	0.25 U/kg	q12h	1-1.5 U/cat	q8-12h
PZI	ProZinc®	40 U/mL – Syringe	0.5 U/kg	q24h (q12h)	1-1.5 U/cat	q12h
NPH	Humulin N® Novolin N®	100 U/mL – Syringe/pen	0.25 U/kg	q12h	1-1.5 U/cat	q8h
Glargine 100 U/mL	Lantus®	100 U/mL – Syringe/pen	0.3 U/kg	q12h	1-1.5 U/cat	q12h
Glargine 300 U/mL	Toujeo®	300 U/mL – Pen	0.5 U/kg	q24h (q12h)	0.5 U/kg	q12h (q24h)
Detemir	Levemir®	100 U/mL – Syringe/pen	0.1 U/kg	q12	1-1.5 U/cat	q12h
Degludec	Tresiba®	100/200 U/mL – Pen	0.5 U/kg	q24h (q12h)	1-1.5 U/cat	q12h

Table 2. Guidelines for starting dose and frequency of administration of various insulin formulations in dogs and cats newly diagnosed with diabetes mellitus.

Ideally, insulin therapy in diabetic dogs should mimic a “basal-bolus” pattern (Figure 1).[10] The basal-bolus regimen involves the use of a basal insulin (typically a long-acting formulation with a flat time-action profile, administered once daily) in combination with a bolus insulin (typically intermediate-acting in dogs, administered at mealtimes).

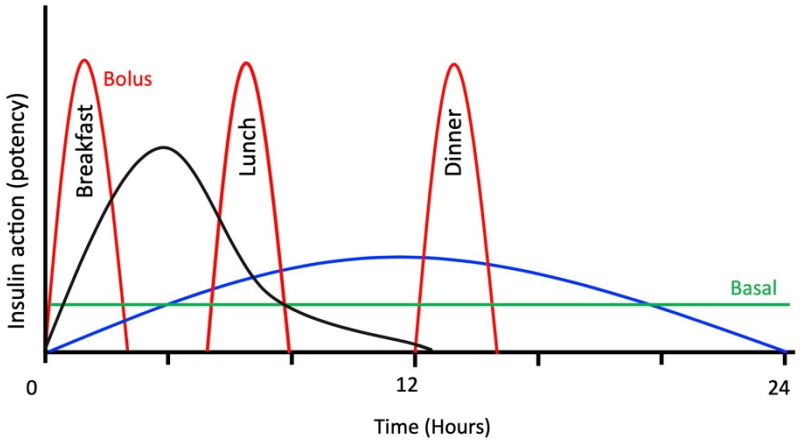

Figure 1. Example of basal-bolus insulin therapy in diabetic people: Red – bolus insulin requirement; Green – basal insulin requirement; Blue – Typical basal insulin kinetics; Black – Typical intermediate insulin kinetics. In: Gilor C, Fleeman LM. One hundred years of insulin: Is it time for smart? Journal of Small Animal Practice. 2022;63(9):645–660.

However, this approach may not always be feasible due to cost and the need for multiple daily injections. The alternative is one of two compromises: (1) selecting a “basal” insulin, or (2) selecting an “intermediate-acting” formulation that is long enough in duration to be administered only once or twice daily, with a curved time-action profile and a peak that is somewhat congruent with peak insulin requirement postprandially.[9] Intermediate-acting formulations, however, are associated with some drawbacks such as the need to match insulin therapy to consistent feeding, marked day-to-day variability, and increased risk of hypoglycemia.[11-15] In the past decades, human diabetology has shifted to using recombinant insulin analogs which are designed to closely mimic physiologic insulin secretion and to have minimal within-day and between-day variability, which is an important feature in minimizing hypoglycemic events.[16,17] Long-acting insulin analogues are modified to enhance their association either as hexamers or through lipophilic interactions, resulting in a slower rate of absorption, a flat ("peakless") pharmacokinetic profile and low day-to-day variability. These formulations are commonly used as "basal" insulins in diabetic humans.[10] Currently, two formulations meet this standard in dogs: insulin glargine 300 U/mL (IGla300, Toujeo®) and insulin degludec (IDeg, Tresiba®).[18,19] Insulin glargine 300 U/mL is a recombinant human insulin analog in which asparagine at position A21 is replaced with glycine and two arginine residues are added to position B30.[16] This synthetic molecule is soluble at a pH of 4 (as supplied) but at physiologic pH (in the subcutaneous tissues) forms microprecipitates, slowing its absorption after injection. In dogs, IGla300 have long duration of action, a relatively peakless pharmacokinetic profile, and low potency.[18] Insulin degludec differs from human insulin in that replacing the B30 amino acid is fatty acid (hexadecanoic acid) that is linked to lysine at B29. Formulated with phenol and zinc, IDeg forms strand-shaped multihexamers in subcutaneous depot that gradually release monomers into circulation as zinc diffuses out of the multimer. Insulin degludec binds to albumin and dissociates slowly prior to insulin receptor binding.[16] In healthy dogs, duration of action lasts more than 20 hours with a flat time-action profile.[19] Despite their potential as “basal” insulin options for diabetic dogs, clinical trials evaluating the use of IGla300 and IDeg are currently limited.

In patients with some residual beta cell function, such as many diabetic cats, administering only a "basal" insulin may lead to complete normalization of blood glucose (BG) concentrations if postprandial endogenous insulin secretion is sufficient to meet the "bolus" requirement and/or if "bolus" requirement is sustained and prolonged.[20] This is especially true in cats, where the postprandial insulin requirement is relatively stable and unchanging throughout the day due to slow transit time, frequent feeding, and/or a diet low in carbohydrates.[20] Among available options, IGla300 is the formulation that most closely meets the "basal" insulin standards in cats.[20]

Continuous glucose monitoring system

In recent years, glucose monitoring has been revolutionized by the development of CGMSs, wearable non/minimally-invasive devices that measure interstitial glucose (IG) concentration almost continuously for several consecutive days/weeks.[21] These devices allows real-time and comprehensive assessment of IG excursions occurring throughout the day and night, as well as of glucose variations over consecutive days, enabling clinicians to make quicker and more informed decisions about insulin dose titration.[21] Continous glucose monitoring systems are nowadays used with increasing frequency to monitor canine and feline diabetic patients and seem to offer a solution to the problem of interpreting a classical 12-hours-BGC and avoiding serial venipuncture. Figure 2 presents a comparison between a hypothetical BGC and CGMS-derived data. Due to their low sampling frequency, BGCs fail to provide a comprehensive glucose profile and, consequently, cannot capture all critical episodes occurring throughout the day. Furthermore, BGCs are unable to detect glucose variability observed across multiple days.

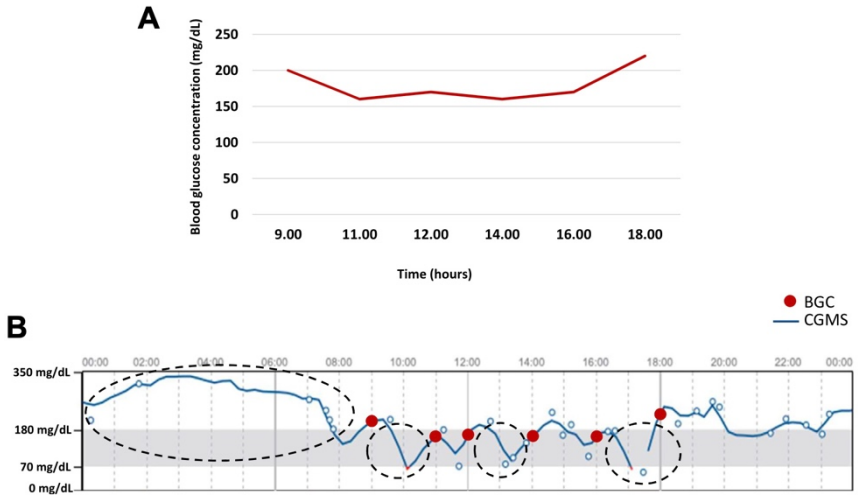

Figure 2. Representative glucose monitoring data obtainable with intermittent measurements of blood glucose using a portable blood glucose meter (A and red circles in B) and using a CGMS (blue line in [B]). Dotted circles in Figure B denote hyperglycemic and hypoglycemic episodes that, using only intermittent PBGM measurements, are not detectable. BGC, blood glucose curve. In: Del Baldo F, Fracassi F. Continuous Glucose Monitoring in Dogs and Cats: Application of New Technology to an Old Problem. Veterinary Clinic of North America: Small Animimal Practice. 2023;53(3):591–613.

The Abbott FreeStyle Libre® is the most commonly used CGMS in veterinary diabetic patients. This device measures IG every minute via a disposable disc-shaped sensor (35 mm [diameter] X 5 mm [height]) with a small catheter inserted under the skin, and it can be worn for up to 14 days. Glucose detection is based on Wired Enzyme Technology that consists of both enzymatic (glucose oxidase) and amperometric (electrodes) systems.[21] The FreeStyle Libre is factory-calibrated and does not require fingerstick BG measurements for calibration.[21] Almost all the studies performed on dogs and cats have evaluated the accuracy of FreeStyle Libre 1.[21] The FreeStyle Libre 2 was developed some years later and a recent study has investigated its accuracy in cats.[22] In 2020, a third generation of the device, FreeStyle Libre 3 (FSL3), was licensed for use in diabetic people.[23] The FSL3 uses the same sensing technology as the FSL2 to measure IG. Like

FSL2, the FSL3 provides continuous IG readings every minute, as well as offering glucose levels, trends and alerts. However, the FSL3 lasts longer (15 days), has a one-piece sensor applicator, and the sensor is about 60% smaller (21 mm X 3 mm) than FSL1 or FSL2.[23] Moreover, the FSL3 automatically sends the results to a smartphone without requiring users to scan the sensor to to obtain a glucose result. In a recent study, the FSL3 demonstrated accurate performance across the dynamic glycemic range in diabetic people. However, no published studies have evaluated the performance of FSL3 in diabetic dogs and cats.

The FreeStyle Libre transfers IG data from the sensor to the FreeStyle LibreLink mobile application, and when the device is connected to the internet, the data is automatically uploaded to the LibreView system. LibreView is a free, secure, cloud-based diabetes management system provided by Abbott, enabling remote data sharing with healthcare providers.[21] The system generates comprehensive glucose reports from the uploaded IG data, including the "daily log" and the "Ambulatory Glucose Profile" (AGP). The daily log display IG trend and fluctuations during the 24-hour periods and is probably the most useful glucose report for making therapeutic decision (Figure 3).

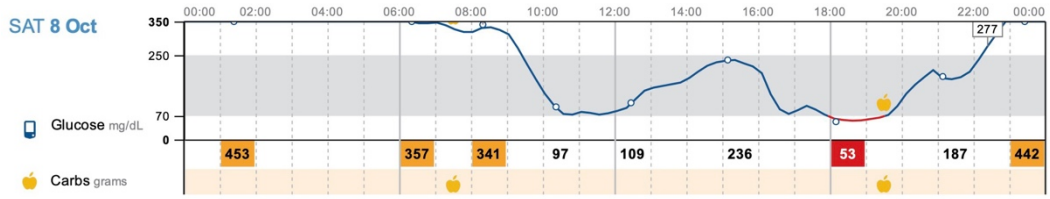

Figure 3. Daily log (AGP) generated by the Libreview System. The interstitial glucose (IG) values detected by the app are reported as numbers and are identified by the empty circles. The red box highlights the IG values < 70 mg/dL, whereas the yellow box highlights IG values > 350 mg/dL. Using the FreeStyle LibreLink mobile app, there is the possibility of adding notes to track food (yellow apple), insulin administration (green box), exercise, and other events (not shown in this figure).

The AGP report (Figure 4) provides both a visual and a statistical summary of the glucose metrics such as mean glucose (MG) and the percentages of time below range (TBR%), time in range (TIR%), and time above range (TAR%), along with glycemic variability expressed as percent coefficient of variation (CV%). In human medicine, these metrics are now regarded as supplementary glycemic targets and outcome measures alongside glycated hemoglobin.[24,25]

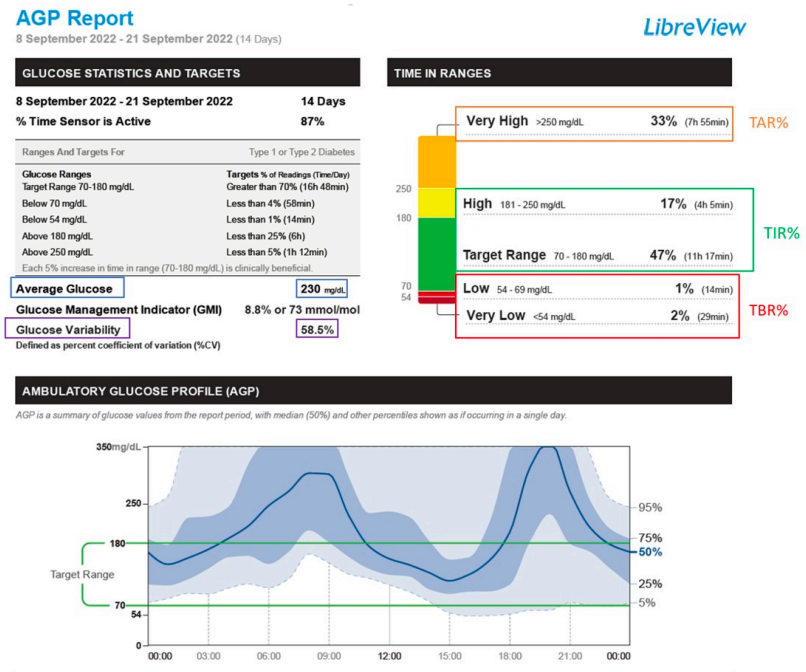

Figure 4. Ambulatory glucose profile (AGP) generated by the Libreview System. TAR%, percent time above range; TBR%, percent time below range; TIR%, percent time in range.

In diabetic veterinary patients, it is advisable to apply the FreeStyle Libre continuously from the time of DM diagnosis until stabilization is achieved, defined as the absence of clinical signs, stable body weight within the ideal range, and blood glucose values between 80–250 mg/dL (Table 3).[26]

Eversense XL® (Senseonics) is an innovative system, monitoring IG in people with DM.[27] The main advantages of Eversense XL are the extended life of up to 180 days, the reduced need for sensor replacement, and the flexibility of being able to remove the external transmitter. This device overcomes some of the limitations of transcutaneous CGMSs, such as trouble inserting the sensor, insertion pain, the burden of frequent sensor replacement, discomfort from wearing the sensor, dissatisfaction with wearing diabetes devices, sensor dislodgement and skin irritation.[27-29] Eversense XL has been shown to be safe and accurate for use in diabetic individuals;[28,29] however, studies in veterinary diabetic patients are rare.

Monitoring veterinary diabetic patients with FreeStyle Libre
Remote re-evaluation every 2–3 days following sensor application ▪ Assessment of interstitial glucose values using the LibreView system ▪ Collection of information on the animal's clinical signs ▪ Adjustment of insulin dose as needed (10% to 25%)
In-hospital re-evaluation two weeks after sensor application (or earlier if a new sensor is needed) ▪ Review of medical history, physical examination, and body weight measurement ▪ Assessment of interstitial glucose values using the LibreView system ▪ Adjustment of insulin dose as needed (10% to 25%) ▪ Application of a new FreeStyle Libre sensor, if required ▪ **Remote re-evaluation every 2–3 days following sensor application**
Re-evaluations in dogs/cats monitored with FreeStyle Libre ▪ Follow the previously outlined protocol until stabilization is achieved (absence of clinical signs, stable body weight, and glucose values between 80 and 250 mg/dL). Thereafter, apply a new sensor approximately every 2–3 months or whenever the dog/cat exhibits clinical signs indicative of inadequate glycemic control.

Table 3. Overview over monitoring protocol used at author's institution in diabetic dogs and cats monitored with FreeStyle Libre.

CANINE HYPOADRENOCORTICISM

Hypoadrenocorticism (HA) is the umbrella term for a range of naturally-occurring or iatrogenic disorders which cause a reduced function of the adrenal cortex and results in a state of glucocorticoid deficiency, mineralocorticoid deficiency or both.[30] In dogs, the majority of cases (>95%) of naturally occurring HA result from primary adrenal gland failure which is thought to be a result of the immune-mediated destruction of the adrenal cortices. A loss of over 90% of adrenocortical function is necessary before clinical signs of glucocorticoid and mineralocorticoid deficiency appear.[31] Secondary hypoadrenocorticism, resulting from reduced ACTH secretion by the pituitary gland, is much rarer. Reduced ACTH levels cause atrophy of the adrenal cortex (sparing the zona glomerulosa) and lead to impaired glucocorticoid secretion, while mineralocorticoid secretion remains unaffected.[31]

In the majority of cases of primary HA, both glucocorticoid and mineralocorticoid secretions are impaired, resulting in hypocortisolemia and electrolyte abnormalities (hyponatremia and hyperkalemia); nevertheless, up to 30% of dogs with primary HA have normal electrolyte concentrations at diagnosis.[32-34] This form of the disease is therefore defined as eunatremic, eukalemic hypoadrenocorticism (EEH), also defined as "atypical" HA.[35] Dogs with EEH are characterized by a condition of permanent hypocortisolemia usually associated with a low to undetectable aldosterone concentration, despite having normal electrolyte concentrations.[36,37]

Clinical signs and clinicopathological abnormalities

Clinical signs of hypoadrenocorticism in dogs are often vague, wax and wane, and none are pathognomonic for the disease. These signs can occur in dogs of any age or breed, although the diseases is mostly diagnosed in middle-age dogs, with an inconsistent female predisposition reported.[38] The diverse clinical manifestations are attributable to the loss of cortisol's essential roles in regulating metabolism, immune function, and gastrointestinal health, as well as the critical function of aldosterone in maintaining sodium balance and fluid homeostasis. Often, sudden signs of volume depletion (hypovolemia, hypotension) associated with weakness, lethargy, and anorexia predominate in dogs with primary hypoadrenocorticism and electrolyte abnormalities.[38] This severe and acute presentation of the disease is also referred to as "adrenal crisis", previously known as "Addisonian crisis".[30] In dogs with EEH, clinical signs tend to be more chronic and typically include vomiting, lethargy, anorexia, and diarrhea. Additionally, "atypical" cases may remain undetected for longer periods due to the absence of typical electrolyte abnormalities. Consequently, hypoadrenocorticism may be mistaken for other disease processes, such as gastrointestinal disorders.

The classic clinicopathologic abnormalities in dogs with HA include hyponatremia, hyperkalemia, azotemia, and absence of a stress leukogram. Less commonly observed findings include non-regenerative anemia, hypochloremia, hypercalcemia, hyperphosphatemia, hypoalbuminemia, hypocholesterolemia, hypoglycemia, elevated liver enzymes, and metabolic acidosis.[31] However, these abnormalities are not consistently present in all cases. While diagnosing a typical case of hypoadrenocorticism is generally straightforward, some dogs with EEH may lack these classic clinicopathologic features, posing a significant diagnostic challenge.[31]

Diagnostic testing

Although most dogs with hypoadrenocorticism have a deficiency of cortisol and aldosterone, routine diagnostic testing relies on measurement of cortisol concentrations. Aldosterone concentrations are less commonly measured because the assay is not routinely run by commercial diagnostic laboratories.[38] Measurement of a resting (basal) cortisol concentration, a simple and cost-effective screening test, is commonly used to rule out hypoadrenocorticism. A serum resting cortisol concentration <2 µg/dL (<55 nmol/L) has excellent sensitivity for diagnosing HA (99.4%-100%).[39-41] However, due to the low specificity of the test (20%-78.2%), up to 33% of dogs with chronic gastrointestinal diseases, but without HA, may have a BSC <2 µg/dL (<55 nmol/L).[39-44] For this reason, urinary corticoid-to-creatinine ratio (UCCR) and cortisol-to-ACTH ratio have been proposed as alternative screening tests for HA in dogs.[42,45,46]

The UCCR offers an integrated measurement of corticoid production over a given time period. Urine cortisol excretion rises or falls in response to adrenal activity, thereby addressing the issue of fluctuations in serum cortisol concentrations. Since creatinine excretion is relatively constant and kidney function remains stable, dividing the urinary cortisol concentration by the creatinine concentration mitigates the impact of urine volume on the interpretation of urinary cortisol levels. The test is safe, easy to perform, relatively inexpensive, and requires only a single urine sample. In a recent study, a UCCR result of ≤ 10 was found to be 100% sensitive and specific for diagnosing HA.[46] In this study, urinary cortisol was measured using a chemiluminescent immunoassay (CLIA) (Immulite 2000 cortisol; Siemens Health Care

Diagnostics Ltd). Unfortunately, recently there was a change in the Immulite 2000 antibody used for cortisol measurement. An initial review by the European Society of Veterinary Endocrinology (ESVE)—Endocrine Quality Assurance, based on >40 canine urine results, suggested that the cortisol values measured with the new antibody were lower (average bias -70%) than the values obtained with the previous antobody (kit before Lot 550).[47] Based on the above findings, the use of the new antibody might result in different diagnostic performances. Therefore, new reference intervals and diagnostic performances should be evaluated using the currently available antibody.

Although serum resting cortisol concentration and UCCR are helpful in ruling out hypoadrenocorticism, it is important to understand that they are not adequate to confirm the diagnosis. The ACTH stimulation test (ACTHst), which assesses adrenal reserve, is considered the gold standard for the definitive diagnosis of hypoadrenocorticism.[38] It is usually performed by blood sampling before and one hour after the intravenous administration of 5 µg/kg of synthetic ACTH. According to the European Society of Veterinary Endocrinology (ESVE) Agreeing Language in Veterinary Endocrinology (ALIVE), the ACTHst is considered diagnostic for HA if pre- and post-ACTH cortisol concentrations are within or less than the bottom quartile of the reference interval for basal cortisol. For example, if the reference interval for basal cortisol concentration is 1.1-4.4 µg/dL (30-120 nmol/L), a post-ACTH cortisol concentration of 1.9 µg/dL (53 nmol/L) or lower is diagnostic for HA.[30] However, previous administration of glucocorticoids (GC), commonly used in dogs with chronic gastrointestinal signs, can lead to suppression of the endogenous hypothalamic-pituitary-adrenal (HPA) axis, potentially resulting in false-positive results on the ACTHst. For this reason, dogs with HA, in particular EEH, represent a diagnostic challenge. Currently, no guidelines exist regarding the required time span until the ACTHst can be carried out after a dog has been treated with different GC formulations. Generally, the degree and duration of suppression of the HPA axis depends on the dose, potency, half-life, and duration of the GC treatment.[48] There are few and limited published studies regarding the duration of HPA axis suppression in dogs receiving systemic GCs. In these studies, HPA axis recovery in dogs treated with systemic GCs is reported to range from a few days to up to seven weeks after GC discontinuation.[49-55] However, the majority of these studies were carried out on healthy experimental dogs and, as such, the possible interference on HPA-axis from concurrent diseases has not been investigated.

The demonstration of high endogenous ACTH concentration can be an objective method to differentiate EEH from false positive results of the ACTHst due to previous GC administration. Dogs with primary HA tipically have extremely high endogenous ACTH concentrations, while dogs with secondary HA have low endogenous ACTH concentration, although this is a rare condition with marginal clinical relevance. To confirm the diagnosis of primary HA it is appropriate to consider the clinical history, the result of the ACTHst while also demonstrating elevated endogenous ACTH concentrations.

Treatment and monitoring

The therapeutic approach to hypoadrenocorticism varies depending on whether the dog presents in adrenal crisis or with clinical signs of chronic but hemodynamically stable disease. In cases of adrenal crisis, the primary objectives of emergency treatment are to address hypotension, hypovolemia, electrolyte imbalances (particularly hyperkalemia), metabolic acidosis, hypoglycemia, and anemia, when present. In this context, it is important to avoid rapid correction of hyponatraemia in order to minimise the risk of osmotic myelinolysis.[30] Once the diagnosis of hypoadrenocorticism has been confirmed and a positive clinical response to parenteral therapy has been achieved, long-term oral glucocorticoid treatment should be started. Oral therapy should only be initiated when the patient is systemically stable, no longer experiencing vomiting, and has regained a good appetite.[31] Prednisone is the glucocorticoid supplement of choice in dogs. The starting dose is 0.5 to 1 mg/kg/day; the dose should then be gradually tapered over several weeks until the lowest

effective dose that controls the clinical signs. The daily dose of prednisolne should be doubled or tripled before known stressful events.[38]

Dogs with electrolyte abnormalities (hyperkalemia or hyponatremia) should also be treated with mineralocorticoid supplementation, such as fludrocortisone or desoxycorticosterone pivalate (DOCP). The latter is the treatment of choice for most dogs with primary HA, with a starting dose of 1.1–1.5 mg/kg administered intramuscularly or subcutaneously.[56,57] The long-term dosage and dosing interval are determined by electrolyte monitoring. Serum electrolytes should be rechecked 10-15 and 25-30 days after injection until the final dose and dosing interval of DOCP are established. The 10 to 15-day follow-up allows for dose titration, while the 25 to 30-day follow-up facilitates adjustment of the required frequency of administration. General recommendations for dose titration and adjustment of the administration frequency are presented in Figure 5. Following this initial dose titration period, the frequency of in-hospital reevaluation can be decreased to once every 3 to 6 months.[31] The goals of chronic treatment are to avoid clinical signs of HA and excessive glucocorticoid supplementation, while ensuring normal or near-normal electrolyte concentrations.[30] The prognosis for dogs with both primary and secondary HA is usually excellent.[31] The most important factor in the long-term response to therapy is owner education. The disease must be carefully described, and owners must be warned of the consequences of apparently mild illnesses. All owners should have glucocorticoids readily available for administration to their dogs during times of stress.[31]

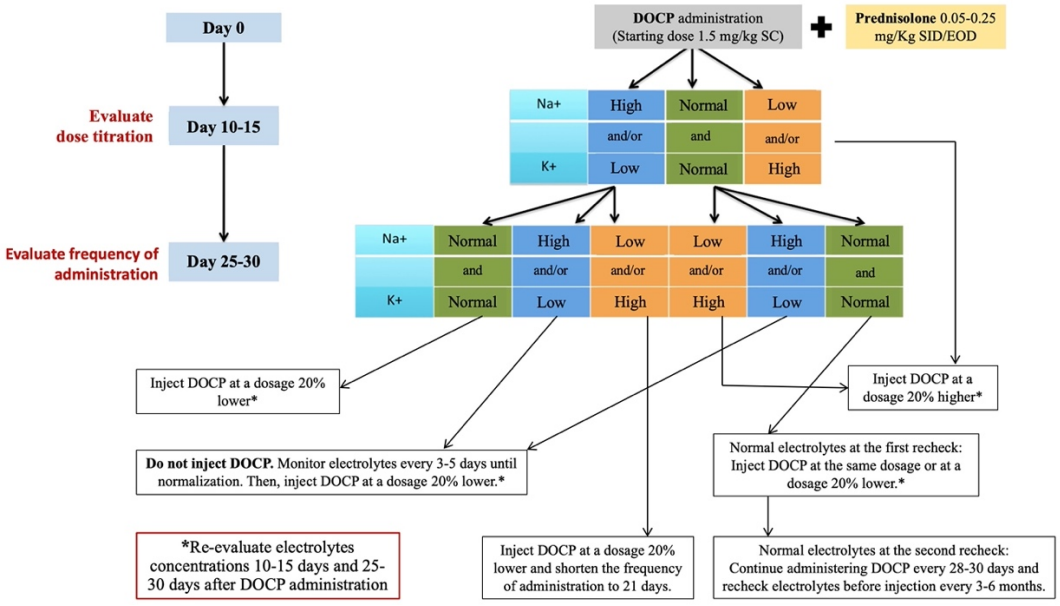

Figure 5. Monitoring protocol for desoxycorticosterone pivalate (DOCP) dose adjustment in dogs with hypoadrenocorticism.

CANINE HYPERCORTISOLISM

Naturally occurring HC or Cushing's syndrome is a prevalent endocrine disorder in dogs, with an incidence of 1–2 cases per 1000 dogs per year.[58,59] According to the ESVE ALIVE project, Cushing's syndrome is the umbrella term for a range of clinical syndromes that is caused by a chronic excess of glucocorticoid activity, which can be due to a range of endogenous or exogenous steroid hormones.[60] In 80–85% of cases, the etiology is attributed to an ACTH-secreting pituitary adenoma, termed pituitary-dependent hypercortisolism (PDH). The increased secretion of ACTH by the pituitary tumor leads to an increased release of cortisol from the adrenal cortex, resulting in a hypercortisolemic state. The remaining 15–20% typically stems from the overproduction of glucocorticoids by either benign or malignant

adrenocortical tumors (adrenal-dependent hypercortisolism). Uncommon causes of HC in dogs include ectopic ACTH syndrome and food-dependent hypercortisolism.[61]

Clinical signs and clinicopathological abnormalities

Spontaneous HC is commonly diagnosed in middle-aged and elderly dogs.[61] Pituitary-dependent hypercortisolism tends to affect smaller dogs more frequently, with approximately 75% of PDH cases occurring in dogs weighing less than 20 kg, while over 50% of cases of adrenal-dependent hypercortisolism (ADH) involve dogs weighing more than 20 kg. A specific breed predisposition has been observed in Poodles, Dachshunds, Bichon Frises, Schnauzers, and Fox Terriers.[62,64] No gender predisposition has been established. The clinical signs of HC result from the combined effects of cortisol, including gluconeogenic, immunosuppressive, anti-inflammatory, proteolytic, and lipolytic actions.[61]

The clinical presentation associated with hypercortisolism (HC) can be highly variable, with some dogs exhibiting multiple clinical signs, while others may be paucisymptomatic. Common signs include polyuria, polydipsia, polyphagia, abdominal enlargement, alopecia, panting, and muscle atrophy.[61] In 10-25% of dogs with pituitary-dependent hypercortisolism (PDH), neurological signs may develop due to the so-called “macroadenoma syndrome”. Compression of surrounding neural structures can result in anorexia or loss of appetite, stupor, circling, ataxia, tetraparesis, head pressing, and seizures.[65]

In both dogs and people, HC has been shown to affect calcium-phosphate homeostasis.[66-70] Dogs with HC exhibit several consequences of altered calcium-phosphate homeostasis, such as calcium-containing urolithiasis, calcinosis cutis, and soft tissue mineralization. It has been reported that dogs diagnosed with HC are ten times more likely to develop calcium oxalate (CaOx) uroliths than individuals of the same breed without the condition.[71] Although the pathogenesis of CaOx urolithiasis remains incompletely understood, the increased urinary calcium excretion observed in dogs with HC is likely a key predisposing factor.[72] Calcinosis cutis refers to the dystrophic deposition of calcium salts in the dermis, epidermis, or subcutaneous tissue, which may affect the temporal region, dorsal midline, neck, and abdomen. The pathogenetic mechanism is not entirely clear, but hypercortisolism is hypothesized to increase gluconeogenesis activity and protein catabolism in collagen fibrils, resulting in the formation of a matrix that attracts and binds calcium ions.[61]

When hypercortisolism is clinically suspected, conducting a comprehensive assessment including a complete blood count, serum biochemistry panel, urinalysis, and blood pressure measurement can provide additional evidence to support the diagnosis. Common abnormalities identified in these tests may include a stress leukogram, elevated serum alkaline phosphatase (ALP) and alanine aminotransferase (ALT) activities, and reduced urine-specific gravity. Proteinuria is observed in more than half of dogs with hypercortisolism and is typically mild to moderate (i.e., urine protein-to-creatinine ratio [UPCR] < 5).[61] While none of these findings are diagnostic on their own, they can collectively support the suspicion of HC.

In dogs with HC, clinicopathological abnormalities associated with disrupted calcium-phosphate homeostasis include hyperphosphatemia, decreased urinary phosphate excretion, and increased urinary calcium excretion.[67-69] Hyperphosphatemia frequently occurs in dogs with HC and represents a negative prognostic factor.[73] Hyperphophatemia is commonly accompanied by elevated serum parathyroid hormone (PTH) concentrations, a condition previously termed adrenal secondary hyperparathyroidism.[68-70] Moreoveor, a recent study reported lower serum 25-(OH)-Vitamin D and plasma fibroblast growth factor-23 (FGF-23) concentrations in dogs with HC compared to controls.[70]

Diagnostic testing

The adrenal function tests are essential for confirming the suspicion of HC and are based on the confirmation of two characteristics: (1) increased cortisol production or (2) decreased sensitivity of the hypothalamic-pituitary-adrenal

axis, as a consequence of negative feedback exerted by glucocorticoids.[61] A major issue when investigating canine HC is that none of the currently available adrenal function tests are totally reliable, with frequent false-positive and false-negative results. Therefore, it is important to test only dogs presenting clinical signs, physical examination findings, and clinicopathologic abnormalities consistent with Cushing's syndrome. This approach maximizes the pre-test probability of hypercortisolism (HC) in the tested population, thereby improving the positive predictive value of adrenal function tests.[74]

Specific endocrine tests are categorized into screening tests and differentiation tests. Screening tests are designed to confirm or rule out the diagnosis of hypercortisolism (HC). These include the UCCR, the ACTHst, and the low-dose dexamethasone suppression test (LDDST). In contrast, differentiation tests are useful for identifying the underlying cause of the condition (PDH vs. ADH). These tests include the LDDST, high-dose dexamethasone suppression test (HDDST) performed on blood or urine, and measurement of endogenous ACTH concentration. In addition to hormonal tests, differential diagnostic methods encompass abdominal ultrasound and advanced imaging techniques, such as computed tomography and magnetic resonance imaging.[61,74]

The UCCR can be used as a screening test for HC, providing an indirect assessment of adrenocortical function. Advantages of this test include its safety, simplicity, and relatively low cost.[61] To minimize the influence of stress, urine for UCCR analysis should ideally be collected at home, at least two days after a veterinary visit. While a urine sample can be obtained at any time of day, morning urine may be preferred, as it typically represents several hours of urine production.[61] The diagnostic sensitivity of the UCCR varies across studies, though it is generally considered high, with reported values ranging from 92% to 100%. Conversely, the specificity of the UCCR for diagnosing HAC ranges from 21% to 100%.[75] Various pre-analytical factors (such as sample collection methods and characteristics of the control population) and analytical factors (such as assay methodology) can impact diagnostic test accuracy. In studies assessing the diagnostic performance of the UCCR, five different assays were used, predominantly radioimmunoassay (RIA).[75] However, chemiluminescent methods offer advantages over RIA, including the elimination of radioisotope exposure, easier easy integration into laboratory processes and rapid turnaround time. As a result, serum and urinary cortisol is commonly measured using a CLIA (Immulite 2000 cortisol; Siemens Health Care Diagnostics Ltd). In one study utilizing CLIA, the UCCR sensitivity and specificity for diagnosing HC were reported to be 92% and 82%, respectively.[76] However, a recent change in the Immulite 2000 antibody used for cortisol measurement has introduced an average bias of -70% in canine urine samples.[47] Consequently, the diagnostic accuracy of UCCR using CLIA requires further investigation.

The diagnosis of HC with the LDDST relies on the demonstration of decreased response of the hypothalamic-pituitary-adrenal axis to negative glucocorticoid feedback.[61] In this test, dexamethasone (0.01 mg/kg) is administered intravenously, and serum cortisol concentrations are measured at baseline and 4 and 8 hours post-administration. A cortisol concentration above the laboratory cut-off at 8 hours post-dexamethasone administration is traditionally considered abnormal and indicative of HC. The advantages of the LDDST include its high sensitivity for diagnosing HC, potential to differentiate between PDH and ADH, and its relative cost-effectiveness. However, disadvantages include lower specificity and the need for 8 hours to complete the test.[61] The diagnostic sensitivity of the LDDST varies between studies, ranging from 85% to 100%.[75] While the reported specifity of the LDDST ranges from 44 to 95%.[75] The majority of commercial laboratories utilize a cut-off of 1.4 µg/dL (38.5 nmol/L) for the 8-hour cortisol concentration, a value extrapolated from RIA studies. However, cortisol concentrations measured can vary between methods and laboratories. Furthermore, these cut-off values may have been derived from studies with various limitations. In a recent study using a CLIA (Immulite 2000), a cut-off point of >1.4 µg/dL (>38.5 nmol/L) for the 8-hour cortisol concentration demonstrated a sensitivity of 85% and a specificity of 100% for diagnosing HC.[77] However, this study was conducted before the

modification of the Immulite 2000 antibody used for cortisol measurement.[47] Therefore, the cut-off points and diagnostic performance of the CLIA should be reevaluated using the currently available antibody.

Several LDDST patterns have been described, including lack of suppression, partial suppression, escape, inverse, and complete suppression (Figure 6). Some of these patterns are used to differentiate PDH from ADH. Two recent studies evaluated the positive predictive value of individual LDDST patterns for diagnosing HAC. Lack of suppression and partial suppresion were the most common pattern in dogs with HAC and were associated with the highest positive predictive values in these population.[78,79] Conversely, a complete suppression pattern was associated with the highest negative predictive value.[78]

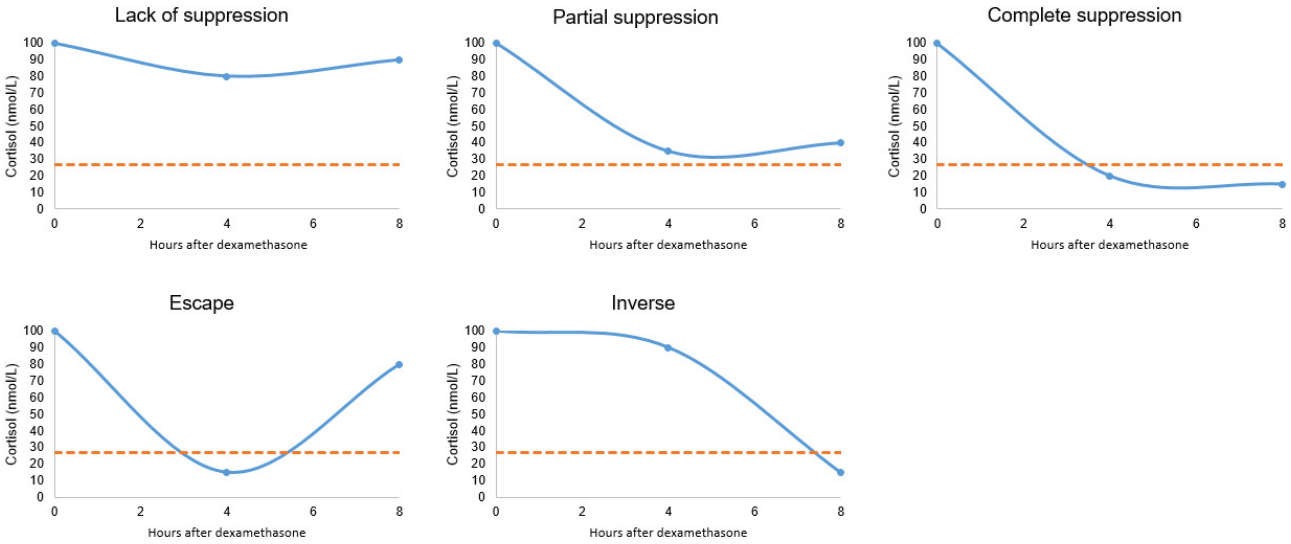

Figure 6: Patterns of the low-dose dexamethasone suppression test in canine Cushing's syndrome. In: Zeugswetter FK, Carranza Valencia A, Glavassevich K, Schwendenwein I. Patterns of the low-dose dexamethasone suppression test in canine hyperadrenocorticism revisited. Vet Clin Pathol. 2021;50(1):62-70.

Treatment and monitoring

The primary goal of treatment of naturally-occurring Cushing's syndrome are optimise quality of life, eliminate clinical signs, and to reduce long-term complications and mortality.[60] These are achieved by eliminating the source of either ACTH or autonomous adrenal hormone excess, or at least, controlling excess adrenal hormone secretion.[60] Treatment ideally should be considered only if there are clinical signs consistent with naturally-occurring Cushing's syndrome and when the disease is confirmed by endocrine testing.[60] Additionally, differentiating between the forms of naturally occurring Cushing's syndrome is essential for optimizing management strategies and prognostic assessments.[60] Treatment strategies and protocols depend on various factors, including the severity and form of the condition (PDH vs. ADH), the presence of hypercortisolism-related complications or concurrent diseases, available therapeutic options, treatment efficacy, potential side effects, and the preferences of both the clinician and the client. Furthermore, considerations such as cost implications and the necessity for frequent follow-up evaluations are of critical importance.

Currently, surgical excision of the causative tumor is the only intervention capable of eliminating excessive ACTH or autonomous cortisol production. However, these procedures carry inherent risks, have limited accessibility, and may not be suitable for all patients. Consequently, medical therapy has become a common strategy for managing clinical symptoms. While surgical intervention is frequently employed for dogs with ADH, most cases of PDH are managed through medical therapies. These therapies typically involve agents that inhibit adrenocortical hormone synthesis, such as trilostane, or those that induce partial or complete necrosis of the adrenal cortices, such as mitotane. Both trilostane

and mitotane, however, are associated with significant adverse effects. Notably, both medications contribute to decreased plasma cortisol levels and increased ACTH secretion, potentially promoting pituitary tumor growth.[80]

Trilostane, a synthetic steroid analog, is the treatment of choice for the medical management of HC. Its mechanism of action involves the competitive and reversible inhibition of the steroidogenic enzyme 3β-hydroxysteroid dehydrogenase, a crucial component in the biosynthesis of all adrenocortical hormones. This inhibition disrupts the production of cortisol and aldosterone, resulting in a cascade of hormonal responses, including an increase in ACTH concentrations due to cortisol suppression.[81] Previous research indicates that trilostane demonstrates an effectiveness range of 67% to 100% in resolving various signs of hypercortisolism within a period of 3 to 6 months for both dogs with PDH and ADH.[82-90] Common clinical manifestations such as polyuria/polydipsia, polyphagia, and lethargy tend to gradually improve within the initial months of trilostane treatment, while resolution of dermatological irregularities may require additional months. Typical laboratory anomalies associated with HC also show improvement with trilostane treatment.[61]

Ensuring effective management of HC with trilostane requires regular and frequent monitoring. Among the commonly employed monitoring methods, the ACTHst is frequently used. This test evaluates the adrenal glands' ability to secrete cortisol in response to stimulation and serves as an indicator of cortisol reserve. Alternative methods recently proposed include measuring cortisol concentration prior to trilostane administration (pre-pill) and assessing haptoglobin concentration.[91,92] However, there is currently no consensus on the gold standard for monitoring trilostane treatment, and it is generally accepted that comprehensive history-taking cannot be replaced by any laboratory variables.[87] Therefore, it is crucial to conduct a thorough assessment of clinical history and physical examination findings to determine any necessary adjustments in the dosage of trilostane.

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Chapter 3 – Canine and Feline Diabetes Mellitus

3.1 | Effect of a homemade diet compared to a commercial diet on glycaemic variability and glycaemic control assessed by continuous glucose monitoring system in diabetic dogs: a randomized crossover trial

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ABSTRACT

Objectives

To evaluate the effects of a homemade diet (HMD) and a commercial diet (CD) on glycaemic control and glycaemic variability (GV) of diabetic dogs monitored with FreeStyle Libre continuous glucose monitoring system (CGMS).

Methods

Prospective randomised crossover study including ten client-owned diabetic dogs on insulin treatment with good glycaemic control. Dogs were randomly assigned to receive either a moderate-fibre (total dietary fibre on a dry matter basis [TDF]: 8.6%) HMD or a high-fibre (TDF: 18%) CD in a 2x6-week period. Dogs were re-evaluated every 2 weeks. Clinical and clinicopathological variables, selected CGMS-derived and GV metrics, glucose nadir, and post-prandial hyperglycaemia were recorded. Differences between diets were analysed by a repeated measure ANOVA fitting a crossover design with pairwise comparisons.

Results

There were no differences in insulin dose and glycaemic control levels between the two dietary period. The HMD significantly reduced serum cholesterol concentration (mean difference: 76; 95% CI: -51.97 to 204). The percentage of time above glucose range (TAR%) was significantly lower (mean difference: 22.5; 95% CI: 1.08 to 43.9) and the percentage of time below range (TBR%) higher (mean difference: -6.9; 95% CI: -12.4 to -1.38) during the HMD period. The percentage of time in range (TIR%) and GV metrics were not different between the two diets.

Clinical significance

The HMD and CD can be considered valid dietary options in diabetic dogs. The results suggest that, with regard to the diets examined, the HMD might have a more effective glucose-lowering effect compared to the CD.

INTRODUCTION

Diet plays a crucial role in the management of dogs with diabetes mellitus (DM).[1] Several pet food companies offer commercial diets (CD) specifically formulated for diabetic dogs. While the composition of these diets varies, most are characterised by moderate to high fibre, high-quality protein, and restricted fat content.[1,2] Additionally, all dry diabetic CD and the majority of wet CD contain digestible "complex" carbohydrates (CHO), primarily in the form of starch.[2] The starch content in pet food can vary significantly, with higher levels typically found in dry products, where starch is essential for the formation of extruded dry kibble.[2] Most studies assessing the role of nutrition in the glycaemic control of diabetic dogs have focused on the effects of dietary fibre and CHO.[3-8] However, there is currently no consensus on recommended types and levels of dietary fibre and CHO in diabetic pet foods. The choice of diet ultimately depends on the weight of the diabetic dog, concurrent diseases, and both owner and dog preferences.[1] Although the majority of pet owners prefer CD, some are interested in providing a homemade diet (HMD) for their animals.[9] The preparation of HMD may enhance owners' sense of involvement with their pets, and anecdotal evidence suggests that this practise is increasing.[10] Home-cooked diets could prove beneficial for dogs with DM, as their nutritional content can be customised to meet the individual patient's needs, and clinical trials evaluating its use in client-owned diabetic dogs are warranted.

The Freestyle Libre (FSL) continuous glucose monitoring system (CGMS) has revolutionised the management of DM in dogs.[11-14] This device enables real-time and comprehensive assessment of glucose trends and time spent within target ranges,[14] allowing clinicians to make faster and more informed decisions about insulin dose titration.[15] Moreover, thanks to CGMS, various metrics assessing glycaemic variability (GV), which refers to glycaemic excursions throughout the day (within-day GV) or on different days (between-day GV), are now affordable. In human medicine, GV is emerging as an additional glycaemic target due to its association with short- and long-term diabetic complications.[16,17] Additionally, in diabetic people, there is growing evidence that GV can be influenced by several nutritional factors, including types and levels of CHO, protein, and fibre content of the diet.[18-22] In veterinary medicine, the concept of GV has gained attention in recent years,[23-26] but there are currently no studies that have evaluated the effects of nutritional factors on GV of diabetic dogs. The aim of this randomised crossover study was to evaluate the effects of an HMD and a CD on glycaemic control and GV of client-owned dogs with stabilised DM, monitored with FGMS. The study was designed to minimise the influence of non-dietary variables and facilitate a thorough evaluation of the effectiveness of nutritional therapy.

MATERIALS AND METHODS

Animals

Diabetic dogs receiving insulin treatment were recruited from 3 referral centres and prospectively enrolled in the study between September 2021 and December 2022. Diagnosis of DM was performed according to the Agreeing Language In Veterinary Endocrinology (ALIVE) criteria established by the European Society of Veterinary Endocrinology (ESVE).[27] Dogs were eligible if they had been diagnosed with DM for at least 3 months, the type of insulin had not been changed in the 30 days preceding admission, and glycaemic control was deemed "stable" (ALIVE Diabetic Clinical Score ≤ 3)[27] at the time of enrolment. Dogs not compliant with the dietary regimen, those with a relevant concurrent disease requiring a specific diet (e.g., renal prescription diet), and dogs that had received systemic or topical glucocorticoids or were diagnosed with diabetic ketoacidosis (DKA) within the previous 30 days were excluded. The trial was approved by the Scientific Ethics Committee of the University of Bologna (protocol number 296279/2021), and informed consent was obtained from each dog owner at the time of enrolment. The recruitment of dogs in the study was voluntary and the only cost for the owners was the purchase of ingredients listed in the HMD recipe, while the CD was provided at no cost.

Diets and study design

The study was a prospective, randomised, crossover study. Using an online software program (Research Randomizer, Computer software, http://www.randomizer.org/), dogs were randomly assigned to one of the two diet periods: CD-HMD or HMD-CD. Each diet was fed for a 6-week period, with a 5-day transition in between, and the crossover design ensured that each dog received the two diets. The CD was a therapeutic veterinary diabetic diet for dogs (Monge Vetsolution Diabetic, Monge & C. SpA, Monasterolo di Savigliano, CN, Italy), while HMD was a home-made diet formulated to be nutritionally complete and designed to be similar to CD in terms of protein, fat and starch content on a dry matter basis. Characteristics and chemical composition of the two experimental diets are shown in Table 1. The HMD was recreated in the laboratory and proximate analyses of the experimental diets (HMD and CD) were conducted according to International Standard methods.[28] Comprehensive instructions on preparing the HMD recipe and determining the daily feeding amount were provided to the owners. The daily feeding amount for each diet was established based on each dog's nutritional needs, ensuring no unintentional body weight changes during the study. To achieve this, the caloric intake from the previous diet was maintained and adapted to the experimental diets. For each diet, the daily amount was divided equally between two meals.

On dry matter basis (%)	CD†	HMD‡
Crude protein	33	35
Ether extract	12	15
Starch	25	26
Ash	9	5
Crude fibre	6.6	2.4
Total dietary fibre	18	8.6
Soluble dietary fibre	2.9	2.0
Insoluble dietary fibre	14.8	6.6
Carnitine (mg/kg)	270	
ME (Kcal/100g)	308	379
Nutrients are expressed % dry matter; ME: metabolisable energy.		

Table 1. Chemical compositions and ingredients of commercial diet (CD) and homemade diet (HMD) fed to diabetic dogs.

†CD: Monge VetSolution Diabetic for dogs. Ingredients: dried chicken meat, tapioca (20%), potatoes (14%), pea fibre, dried fish (anchovy), dried eggs, salmon oil, dried duck meat, brewers' yeast, minerals, chicken oil, Xylo-Oligosaccharides (XOS 0.4%), fenugreek saeed (0.15%), products and by-products from processing fresh fruits and vegetables (melon juice concentrate – Cucumis melo cantalupensis – source of superoxide dismutase 0.005%), milk protein powder. L-carnitine (260 mg/kg).
‡HMD: Homemade diet. Ingredients: Fresh chicken breast, pearled barley, peas, potatoes, lard, vegetable oils (sunflower, wheat), minerals and vitamins supplement (Essential Cane Adult, Chemivit), salmon oil (EPA+DHA 31%), calcium carbonate, L-carnitine.

Evaluations

Baseline data were collected at the time of inclusion in the study (T0) and re-evaluations were performed every 2 weeks (T2-T4-T6 for each dietary period) thereafter. At T6, the dietary regimen was changed (e.g., from CD to HMD, and vice versa). During each evaluation, the following were performed: recording of ALIVE Diabetic Clinical Score[27] based on owner perception of clinical signs, body weight, body condition score (BCS), clinical hypoglycaemic events and unusual clinical signs (e.g., vomiting or diarrhoea) in the previous two weeks; assessment of IG data and application of a new Freestyle Libre® sensor; insulin dose adjustment. Owner perception of clinical signs and assessment of IG data informed insulin dose adjustments and final categorisation into level of glycaemic control (maximum score 12: 0-3 good control, 4-8 moderate control, 9-12 inadequate control).

Blood and urine samples were collected at baseline (T0) and at the end of each 6-week dietary period (T6). At the time of blood collection, the dogs had to be fasted for at least 12 hours. Measurement of serum fructosamine concentrations (Fructosamine 17350H, Sentinel Diagnostic, Milano, Italy),[29] Chemistry profile (AU 480, Beckman

Coulter/Olympus, Brea, CA) and urinalyses were performed by standard laboratory methods at the internal laboratory of the Veterinary Teaching Hospital of the University of Bologna.

Continuous glucose monitoring system

The IG measurements were acquired with a validated CGMS (FreeStyle Libre, Abbott Laboratories Ltd, Chicago, Illinois).[19] In this study, sensor placement was performed as previously described.[19] More than one generation of Freestyle Libre (i.e., Freestyle Libre 1 and Freestyle Libre 2) were used throughout the study. At each time point, FSL-derived metrics including mean glucose (MG), percentage of time-in-range (TIR%, 70-250mg/dL), time above range (TAR%, >250mg/dL), and time below range (TBR%, <70mg/dL) were recorded. The following GV metrics were computed by processing FSL data in a web-based application (GlyCulator 3.0)[30]: standard deviation of mean glucose concentration (SD), within-day percent coefficient of variation (CV%), between-day CV%, and mean amplitude of glycaemic excursion (MAGE). Interstitial glucose concentrations were analysed for post-prandial hyperglycemia (PPH; 30, 60, 90 and 120 minutes after meal) and glucose nadir.

Data Analysis

Statistical analysis was performed using commercial statistical software packages (GraphPad Prism 9.5.1, San Diego, California). Descriptive statistics were generated to characterise the study population. The continuous data were assessed using the Shapiro-Wilk's test for normality and reported as mean ± SD or median and range (minimum and maximum value), depending on whether the data were normally or not normally distributed, respectively. Mean differences and their 95% CI were computed. Categorical variables were described with frequencies, proportions, or percentages. Differences between variables were tested by a generalised linear model (GLM) fitting a cross-over design with diet (CD and HMD) and period (CD-HMD or HMD-CD) as fixed factors, and subjects as random factors; period and diet x period interaction were tested to exclude any carry-over effect. Post-hoc analysis within the framework of the GLM was conducted using pairwise tests. Differences between the two experimental diets for the clinicopathological variables measured at the inclusion (T0) and at the end of each 6-week dietary period (T6) were assessed using Wilcoxon Rank-Sum test or paired T-test. Statistical significance was set at $P < 0.05$.

RESULTS

Study population

A total of 10 client-owned diabetic dogs were included in the study. The majority were purebred dogs (6/10: Miniature poodle, Deutsch Drahthaar, Epagneul Breton, Jack Russel terrier, Labrador retriever, Cavalier King Charles Spaniel). Six spayed female and 4 males, one of which was intact, were included. At the time of enrolment, the median age was 9 years (range, 2-14), the median body weight (BW) was 10 kg (5.2-36.5), and the median BCS was 5/9 (3/9-7/9). Dogs were treated with insulin glargine 300 U/ml (6/10 Toujeo, Sanofi-Aventis Deutschland GmbH) or porcine lente (4/10, Caninsulin, Intervet International B.V.). All dogs receiving lente insulin were managed with twice daily insulin injections, while dogs receiving insulin glargine 300 U/mL were managed with once (in 3/6 dogs) or twice (in 3/6 dogs) daily insulin injections. Insulin type was not changed during the study. Only one dog had a concurrent disease, myxomatous mitral valve disease (ACVIM stage B1). Concurrent medications were recorded in one dog, which was receiving bezafibrate (Bezalip, Aurobindo Pharma, Italy), and the treatment was continued throughout the study without any dosage changes.

Clinical and clinicopathological outcomes

All dogs were compliant with the two dietary regimens, and none developed adverse effects. Body condition score did not significantly change throughout the study and between the two diets ($P = 0.78$). Median BW fluctuated between 10.13 kg to 10.7 kg (min: 5.1-5.45 kg; max: 34.9-36.5 kg), without significative changes ($P = 0.69$). As per

inclusion criteria, all dogs enrolled had good diabetic control as assessed by the ALIVE score (median: 2, range: 0-3). When HMD was fed, 10/10 dogs had good glycaemic control at T6 (Median ALIVE score 3, range 0-3); while, when CD was fed 9/10 dogs had good glycaemic control and 1/10 had moderate control (Median ALIVE score 3, range 0-4). However, differences between the two dietary period were not significant ($P = 0.87$; Figure 1). The median (range) insulin dose at the time of inclusion (baseline) was 1,1 (0.55-1.9) U/kg/day. While the median (range) insulin dose at T6 was 0.97 (0.54-2.53) U/kg/day in CD dogs and 0.95 (0.44-2.43) U/kg/day in HMD dogs. There were no differences in insulin dose between the two dietary period ($P = 0.32$).

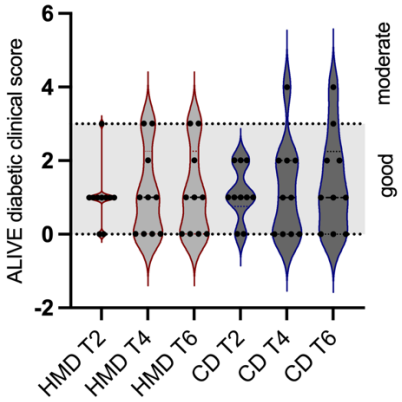

Figure 1. Violin plots showing the ALIVE diabetic clinical score recorded in 10 diabetic dogs (black dots) receiving both a commercial diet (CD) and a homemade diet (HMD) during periods of 6 weeks each, with follow-up recorded every 2 weeks (T2, T4, and T6). The grey shaded area represents the range from 0 to ≤ 3, indicating good glycaemic control. In the violin plot, coloured dotted line represents quartile, black dotted line represents the median.

Clinicopathological variables assessed in diabetic dogs at baseline and at the end of each dietary period (T6) are shown in Table 2. Dogs receiving the CD had significantly higher serum cholesterol concentration at T6 compared to baseline (mean difference: -45.3; 95% CI: -90.3 to -0.33; $P = 0.048$). When fed HMD, dogs had lower cholesterol level at T6 compared to CD (mean difference: 76.2; 95% CI: 18.36 to 134; $P = 0.02$), while other parameters were not different between the two diets.

Variable	Reference	Baseline	CD (T6)	HMD (T6)	*P* value
Fructosamine (µmol/l)	222-382	547±56	549±56	497±93	0.07
Cholesterol (mg/dl)	123-345	337±133*	382±142*	306±131	0.02
Triglycerides (mg/dl)	30-120	54 (43-583)	70 (43-269)	58 (29-321)	0.92
Creatinine (mg/dl)	0.75-1.40	0.83±0.17	0.88±0.12	0.88±0.18	0.94
ALT (U/L)	15-65	69 (42-115)	69 (40-436)	80 (42-219)	0.63
β-HBA (mmol/l)	0-0.9	0.04 (0.02-0.3)	0.03 (0.01-0.1)	0.03 (0.01-0.07)	0.63
Total protein (g/dl)	5.60-7.30	6.31±0.36	6.41±0.52	6.3±0.44	0.39
Albumin (g/dl)	2.75-3.85	3.08±0.26	3.15±0.33	3.10±0.3	0.13
USG	<1.030	1.049 (1.035-1.060)	1,050 (1.032-1.064)	1.040 (1.020-1.054)	0.19
UPC	0-0.5	0.14 (0.09-0.24)	0.13 (0.09-0.45)	0.14 (0.07-0.31)	0.31
Glycosuria (mg/dl)	absent	1000 (0-1000)	1000 (0-1000)	1000 (0-1000)	>0.99
Ketonuria (mg/dl)	absent	2.5 (0-15)	2.5 (0-15)	0	0.25

The asterisk indicates statistically significant differences between baseline and T6 measured by T-test. The *P* values indicate the results of T-test between CD and HMD at T6.
ALT, alanine aminotransferase; β-HBA, β-hydroxybutyrate acid; USG, urinary specific gravity; UPC, urine protein:creatinine ratio.

Table 2. Clinicopathological variables measured in 10 diabetic dogs at the time of inclusion in the study (baseline) and at the end of each 6-week dietary period (T6, CD or HMD). Data are expressed as mean±SD or median (range).

Freestyle Libre Data Analysis

A total of 59219 IG concentrations were recorded. Freestyle Libre-derived and GV metrics assessed in diabetic dogs during each dietary period (T2, T4, and T6) are shown in Table 3. The TAR% was significantly lower at T4 (mean difference: 22.5; 95% CI: 1.08 to 43.9; *P*=0.04, Figure 2C) and TBR% higher at T6 (mean difference: -6.9; 95% CI: -12.4 to -1.38; *P*=0.02, Figure 2D) during the HMD period; however, the MG (Figure 2A) and TIR% (Figure 2B) were not different between the two diets (*P*= 0.07 and *P*=0.10, respectively). No differences in GV metrics were found between the two diets. The PPH 30 minutes after meal tended to be lower (*P*=0.056) in dogs receiving HMD, while no differences were found for the other time points. Glucose nadir did not differ between HMD and CD (*P* = 0.31).

Variable	Diet	T2	T4	T6	*P* value
Mean glucose (mg/dL)	HMD	240 ± 57	207 ± 39	212 ± 52	0.07
	CD	256 ± 50	263 ± 55	252 ± 45	
TIR%	HMD	48.6 ± 19.6	56.1 ± 10.1	52.9 ± 15.9	0.10
	CD	42.1 ± 14	40.3 ± 17.5	46.5 ± 14	
TAR%	HMD	45.2 ± 22.1	32.7 ± 13*	36 ± 20.5	0.04
	CD	52.9 ± 19.6	55.2 ± 21*	49.3 ± 15.5	
TBR%	HMD	6.2 ± 6.6	11.2 ± 6.7	11.1 ± 8*	0.03
	CD	5 ± 7.3	4.5 ± 5.3	4.2 ± 3.5*	
SD	HMD	86.5 ± 23	88.2 ± 23	85.3 ± 20	0.47
	CD	86.1 ± 16	74.4 ± 29	84.2 ± 15	
Within-day CV%	HMD	43.8 ± 8.4	43.8 ± 8.4	43.6 ± 13.2	0.09
	CD	36.6 ± 10.8	34.5 ± 11.3	36.5 ± 9.5	
Between-day CV%	HMD	47.3 ± 10	53.8 ± 8.3	51.9 ± 14.6	0.09
	CD	43.9 ± 13.9	43.2 ± 14.7	43.8 ± 10.1	
MAGE (mmol/l)	HMD	9.76 ± 3.0	10.2 ± 3.4	9.82 ± 3.2	0.83
	CD	9.62 ± 3.1	9.23 ± 2.8	9.29 ± 2.7	

The *P* value refers to the result of the generalised linear model, while the asterisk indicates the timepoint at which statistically significant differences between diets were observed by pairwise comparisons. CD, commercial diet; CV, coefficient of variation; HMD, homemade diet; MAGE; mean amplitude of glycaemic excursion; MG, mean glucose; SD, standard deviation of mean glucose concentration; TAR, time above range (>250mg/dL); TBR, time below range (<70mg/dL); TIR, time-in-range (70-250mg/dL).

Table 3. Freestyle Libre-derived and glycaemic variability metrics obtained in 10 diabetic dogs during each 6-week dietary period (T2, T4 and T6). Data are expressed as mean ± SD.

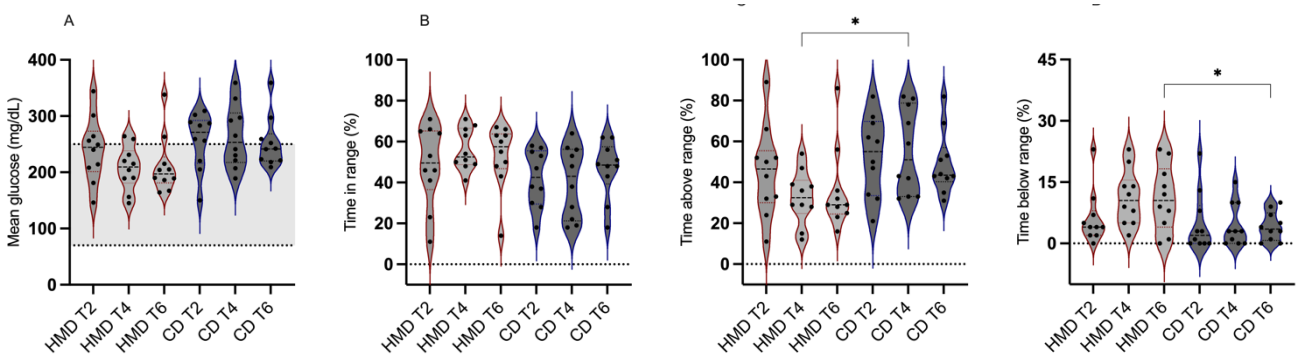

Figure 2. Violin plots showing: (A) the mean glucose; (B) the time in range (TIR %); time above range (TAR%); (D) time below range (TBR%), in 10 diabetic dogs (black dots) receiving both a commercial diet (CD) and a home-made diet (HMD) during periods of 6 weeks each, with follow-up recorded every 2 weeks (T2, T4, and T6). The asterisk indicates significant difference ($P < 0.05$) by pairwise test between HMD and CD. The grey shaded area represents the glucose range between 70 and 250 mg/dL.

DISCUSSION

Nutritional management plays a crucial role in the long-term management of diabetic dogs. However, no consensus exists regarding the ideal composition and macronutrient balance in a dietary formulation for DM in dogs. The nutritional management of diabetic dogs has traditionally relied on commercially available diets, while alternative nutritional strategies, such as home-cooked diets, have not been previously investigated in research studies. This gap in knowledge highlights the importance of investigating whether specific dietary interventions could improve management and potentially enhance the clinical outcome of canine diabetic patients. In our study, diabetic dogs fed an HMD showed similar exogenous insulin requirements and glycaemic control levels as when fed a therapeutic veterinary diabetic dry CD. Therefore, our results suggest that the two dietary formulations used in this study are unlikely to result in clinically relevant differences when fed to individual diabetic dogs. Several hypotheses can be considered to explain our findings: 1) we included dogs with well-controlled diabetes, carefully monitored, making it difficult to detect any worsening of clinical signs or significant changes in insulin dosage; 2) exogenous insulin likely has a predominant effect on glucose homoeostasis in diabetic dogs, potentially masking any subtle dietary influences on glycaemic control; or 3) the methods used to assess glycaemic control and insulin requirements may not have been sensitive enough to detect differences between the diets. For a more precise comparison between the two diets, we also evaluated metrics derived from the FreeStyle Libre®, which is currently the most widely used CGMS in veterinary medicine.[14] This device transfers IG data from the sensor to the FreeStyle LibreLink mobile application, and when the device is connected to the internet, the data is automatically uploaded to the LibreView system. LibreView is a free, secure, cloud-based diabetes management system provided by Abbott, enabling remote data sharing with healthcare providers.[14] The system generates comprehensive glucose reports from the uploaded IG data, including the Ambulatory Glucose Profile (AGP). The AGP report provides both a visual and a statistical summary of the glucose metrics such as MG, TIR%, TAR%, and TBR%, along with glycaemic variability expressed as CV%. In human medicine, an international panel of CGMS experts recently developed consensus guidelines to provide clinicians, researchers, and individuals with DM with standardised recommendations for using, interpreting, and reporting CGMS-derived metrics in routine clinical care and research.[31,32] These metrics are now regarded as supplementary glycaemic targets and outcome measures alongside glycated haemoglobin.[31,32] Although the FreeStyle Libre is increasingly used in diabetic dogs, its integration into routine clinical practise remains limited due to the absence of standardised guidelines for data interpretation. In our study, dogs receiving the HMD showed a reduced TAR% at T4 and an increased TBR% at T6 compared to those on the CD. These findings may suggest a more pronounced

glucose-lowering effect in dogs fed the HMD. This could be attributed to differences in the ingredients and cooking processes between the two diets. The starch source varied between the diets (tapioca meal vs. pearl barley), and it is known that starch plays a key role in modulating glucose fluctuations and lipidemia in diabetic dogs.[34] Therefore, nutritional adaptations for diabetic dogs include low carbohydrate levels to reduce the magnitude of increase of postprandial blood glucose.[35] However, the role of starch in the management of diabetic dogs has not been thoroughly addressed in studies so far, and only recent investigations have focused on it.[33,34,36] Based on these studies, sorghum- and barley-based diets, as well as the inclusion of legumes such as lentils and peas, have been shown to induce better glycaemic responses in insulin-treated dogs compared to rice- or corn-based diets. These ingredients may contain components that help minimise postprandial hyperglycemia in diabetic dogs, such as β-glucan in barley,[33,34] and the high resistant starch content in peas and lentils.[36] Barley and other cereals with a high content of dietary fibers or amylose have a low glycaemic index[37], a concept from human medicine that has some applicability to dogs.[38] The formulation of pet food is a more complex process than preparing a home-cooked diet, because the extrusion process involves the interaction of different components besides their physical and chemical transformations. It is known that not only the type and source of starch, but also the different dietary components of the pet food have an impact on gelatinisation itself and the temperature at which gelatinisation occurs.[39] Contrary to pet food production, preparing a home-cooked diet requires different ingredients to be cooked separately. The interactions between ingredients, e.g., starch-lipid interactions or protein agglomeration, might impact the degree of starch gelatinisation in pet food but not in home-cooked diets.[40] On the other hand, the cooking process of the HMD requires lower temperatures than the extrusion. This study did not standardise the cooking time of barley since the owners were instructed only to cook it until tender with a slightly chewy texture. Moreover, after boiling, barley from the HMD may have preserved some resistant starch and reduced starch gelatinisation, potentially leading to decreased glucose absorption from the gastrointestinal tract.[33] Additionally, the method of preserving barley potentially used by dog owners to avoid daily cooking (refrigeration at 4°C), may have influenced starch retrogradation. These hypotheses may also explain the tendency toward lower post-prandial hyperglycemia observed in the HMD group.

In our population of well-controlled diabetic dogs, the mean TIR% was not different between the two diets and ranged from 40% to 56% when fed the CD and HMD, respectively. Notably, in people with type 1 and type 2 diabetes, it is recommended that over 70% of IG measurements fall within the target range (70-180 mg/dL).[31] Our findings suggest that well-controlled diabetic dogs may not meet the glycaemic targets recommended for diabetic people. However, establishing glycaemic target guidelines for diabetic dogs is beyond the scope of this study, and further research is warranted to assess this aspect.

In diabetic people, GV can be influenced by various nutritional factors, including the types and quantities of CHO, protein, and dietary fibre.[18-22] In this study, we analysed various metrics to assess GV throughout the day (SD, within-day CV%, and MAGE) and during different days (between-day CV%). We found no significant differences between the two diets, which was unexpected given that the consistency and reproducibility of the diet are generally more challenging with HMD compared to CD. In a study evaluating pet owner perceptions regarding the use of HMDs, 30% of pet owners reported that they changed the diets, 40% did not adequately control the quantities of provided ingredients, and 56% indicated that their dog refused to eat at least one food item.[41] In our study, we included highly motivated owners, and every re-evaluation was attended by a nutritionist who verified the proper administration of all the ingredients of HMD and the daily amount of CD fed by the owners. The results of this study underscore the importance of considering owner commitment when using an HMD for the long-term management of diabetic dogs.

Increasing the dietary fibre content is advantageous for managing overweight patients and is thought to enhance glycaemic control in diabetic dogs.[1] However, studies evaluating the effect of different fibre sources on diabetic dogs are still scarce. The diets used in this study markedly differed in the fibre content, with lower levels of total dietary fibre and insoluble fibre in the HMD, and to a lesser extent, soluble fibre. This can be attributed to the challenge of incorporating high amounts of insoluble fibre-rich ingredients, which may negatively affect the palatability of HMD recipes. The ability of the soluble fraction of dietary fibre to form a viscous gel is critical, as it hinders the convective transfer of glucose and water to the intestinal absorptive surface, thereby slowing intestinal glucose absorption. Rapidly fermentable viscous soluble fibres (e.g., gums, pectin) impede glucose diffusion more effectively than insoluble fibres (e.g., cellulose, hemicellulose), making them more beneficial for glycaemic control.[1] In a previous study, diabetic dogs were fed, in a randomised model, dry diets differing in fibre and soluble fraction content. Dogs fed a diet high in fibre (73 g/Mcal) with low soluble fraction (<0.1g/Mcal) had the best outcome in terms of glycaemic control.[42] These findings were not confirmed by another study, in which wet diets differing in fibre content and source, as well as carbohydrate content, did not result in differences in terms of glycaemic control in diabetic dogs.[7] Comparing these outcomes is challenging, as the diet highest in fibre in the study by Fleeman et al (2009)[7] is not comparable to that of Kimmel et al (2000)[42] in terms of total dietary and soluble fibre content, given that the former study used wet rather than dry diets. Therefore, the optimal fibre content and type for diabetic dogs has yet to be established, and individual factors such as body condition score and owner and dog preferences should also be considered. In this study, despite the difference in fibre types and content, body weight and BCS showed no significant changes when diabetic dogs were fed HMD or CD. This raises the question of whether high-fibre diets should be routinely recommended in diabetic dogs.

In this study, when fed HMD compared to CD dogs showed a reduction in serum cholesterol concentrations. The CD and the HMD had a similar fat content, with HMD being slightly higher on a dry matter basis compared to CD. Therefore, other factors than fat content might be involved in the cholesterol-lowering effect exerted by HMD. A possible explanation can be found in the presence of barley in HMD, because similar results have been previously reported in diabetic dogs fed diets containing this cereal.[34] Barley in fact contains ß-glucans that interact with lipids and biliary salts in the bowel and consequently reduce cholesterol levels in humans.[43] However, this mechanism cannot be confirmed in this study and in that of Teixeira et al, (2020),[34] as the fecal bile acids excretion was not measured.

The present study has several limitations, including the small sample size. Additionally, only dogs with well-controlled diabetes were included to minimise the influence of non-dietary variables and allow for a thorough evaluation of the effectiveness of nutritional therapy. However, this selection criterion may have introduced bias into the results. Furthermore, it was not feasible to ensure identical compositions between the two diets, particularly concerning fibre content. Finally, the diabetic dogs were managed at a referral centre, and the experimental conditions of this study may not be replicable in everyday veterinary settings.

In conclusion, both the CD and the HMD can be regarded as valid dietary options for managing DM in dogs. The HMD was associated with a significant reduction in the TAR% and cholesterol, and an increase in the TBR%. These results suggest that the HMD formulated for this study may have a more effective glucose-lowering effect compared to the CD. In contrast, glycaemic variability metrics did not demonstrate significant differences when diabetic dogs were fed either the CD or the HMD. Further research is needed to confirm these findings and explore the long-term effects of the HMD on glycaemic control and overall health in diabetic dogs. Future studies should aim to include larger sample sizes and diverse populations to enhance the generalisability of the results and ensure that these dietary options can be effectively integrated into clinical practise for optimal diabetes management in canine patients.

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3.2 | A dose titration protocol for once-daily insulin glargine 300 U/mL for the treatment of diabetes mellitus in dogs

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ABSTRACT

Background

In purpose-bred dogs, insulin glargine 300 U/mL (IGla300) has long duration of action, peakless time-action profile, and low potency, making it suitable for use as a basal insulin.

Hypothesis/Objectives

To evaluate IGla300 in client-owned diabetic dogs monitored using a flash glucose monitoring system (FGMS).

Animals

Ninety-five client-owned diabetic dogs, newly diagnosed or previously treated with other insulin formulations, with or without concurrent diseases.

Methods

Prospective multi-institutional study. Clinical signs and standardized assessment of FGMS data, using treatment and monitoring guidelines established a priori, guided dose adjustments and categorization into levels of glycemic control.

Results

Initial IGla300 dose was 0.5 U/Kg q24h for newly diagnosed dogs and (median dose [range]) 0.8 U/Kg (0.2-2.5) q24h for all dogs. Glycemic control was classified as good or excellent in 87/95 (92%) dogs. The IGla300 was administered q24h (1.9 U/kg [0.2-5.2]) and q12h (1.9 U/kg/day [0.6-5.0]) in 56/95 (59%) and 39/95 (41%) dogs, respectively. Meal-time bolus injections were added in 5 dogs (0.5 U/kg/injection [0.3–1.0]). Clinical hypoglycemia occurred in 6/95 (6%) dogs. Dogs without concurrent diseases were more likely to receive IGla300 q24h than dogs with concurrent diseases (72% versus 50%, respectively; P =.04).

Conclusions and clinical importance

Insulin glargine 300 U/mL can be considered a suitable therapeutic option for once-daily administration in diabetic dogs. Clinicians should be aware of the low potency and wide dose range of IGla300. In some dogs, twice-daily administration with or without meal-time bolus injections may be necessary to achieve glycemic control. Monitoring with FGMS is essential for dose titration of IGla300.

INTRODUCTION

Insulin treatment is the cornerstone of diabetes mellitus (DM) management in dogs. Ideally, insulin treatment in dogs should mimic the physiology of endogenous insulin secretion, which is characterized by a "basal-bolus" pattern.[1] However, to minimize costs and the need for multiple daily injections, insulin treatment in dogs traditionally has relied on the use of intermediate-acting insulin suspensions administered at the time of feeding.[2] These formulations however are associated with some drawbacks such as the need to match insulin administration to consistent feeding, marked day-to-day variability, and increased risk of hypoglycemia.[3-7] In the past, diabetology in humans has shifted to using recombinant insulin analogs which are designed to closely mimic physiologic insulin secretion and to have minimal within-day and between-day variability, which are important features in minimizing hypoglycemic events.[8,9]

Insulin glargine is a recombinant human insulin analog in which asparagine at position A21 is replaced with glycine and 2 arginine residues are added to position B30.[8] This synthetic molecule is soluble at a pH of 4 (as supplied) but at physiologic pH (in the SC tissues) forms microprecipitates, slowing its absorption after injection.[10] Insulin glargine 300 U/mL (IGla300; Toujeo, Sanofi-Aventis, Bridgewater, New Jersey) is biochemically identical to insulin glargine 100 U/mL (IGla100) but is 3 times more concentrated,[11] which results in lower potency, longer duration, and a flatter time-action profile compared to IGla100.[12] Several studies in people have shown that IGla300 is superior to IGla100 in maintaining glycemic control while decreasing day-to-day variability and frequency of hypoglycemia.[11,13-16] In dogs, IGla300 was shown to have long duration of action, a relatively peakless time-action profile, and low potency.[17] In dogs with toxin-induced DM, IGla300 administered twice daily, showed lower day-to-day variability compared with lente insulin.[7] These properties make IGla300 a good candidate for use as basal insulin in dogs, and clinical trials evaluating it in client-owned diabetic dogs are warranted.

The Freestyle Libre flash glucose monitoring system (FGMS) has revolutionized the management of DM in dogs.[18-25] The device measures interstitial glucose concentrations (IG) on a minute-by-minute basis for up to 14 days, via a disc-shaped sensor with a small catheter inserted under the skin. The FGMS does not require calibration, is accurate, and well tolerated by dogs.[18] Recent studies have demonstrated that the FGMS allows more accurate identification of glucose nadirs, postprandial hyperglycemia, hypoglycemic episodes, and day-to-day variations in glycemic control compared with serial blood glucose curves (BGCs).[20-24] These advantages might be particularly evident when monitoring dogs treated with basal insulin such as IGla300.

Our study was designed to evaluate the feasibility of a treatment protocol using IGla300 as basal once-daily insulin in client-owned diabetic dogs with FGMS monitoring used for dose titration.

MATERIALS AND METHODS

Our study was designed as a prospective multi-institutional data collection study using guidelines established *a priori* for a novel insulin treatment and dose titration protocol. Data were collected from 3 referral centers (Animal Diabetes Australia, Victoria, Australia; Department of Small Animal Clinical Sciences of the University of Florida, Gainesville, Florida, USA; Veterinary Teaching Hospital of the University of Bologna, Bologna, Italy) from January 2021 to January 2023. The timeframe of the study was not predetermined, and we chose to stop case inclusion when the number of cases was sufficient to meet the objectives of our study. Data were generated from routine clinical cases in which IGla300 was used to treat DM and FGMS monitoring was used for dose titration. Attending clinicians at the 3 institutions were asked to enter data into a shared spreadsheet on Google Drive. Before patient recruitment, 3 of the authors (FLM, FF, GC). developed detailed treatment and monitoring guidelines for IGla300 dose titration based on clinical experience and previously published data.[7,17] These *a priori* guidelines were followed by all attending clinicians throughout the study period under the supervision of 1 of the authors (FLM, FF, GC) at each institution. However, throughout the study period

and for all patients included in the study, treatment and monitoring decisions were at the discretion of the attending clinician with complete disregard to the contemporaneous data collection. The trial was approved by the Scientific Ethics Committee of the University of Bologna (protocol number 101123/2023) and by the University of Florida Institutional Animal Care and Use Committee (protocol number 202300000146). This research received no external funding.

Diabetes diagnosis and inclusion criteria

Diagnosis of DM was performed according to the Agreeing Language In Veterinary Endocrinology (ALIVE) criteria established by the European Society of Veterinary Endocrinology (ESVE).[26] Client-owned dogs, both newly diagnosed or previously treated with other insulin formulations, were included. Dogs having already received insulin were transitioned to IGla300 if their DM was poorly controlled or if owners expressed desire to minimize the number of daily injections (i.e., using a long-acting insulin formulation once-daily). Dogs treated with corticosteroids or progestagens and dogs with concurrent acute (e.g., diabetic ketoacidosis [DKA]) and chronic (e.g., Cushing's syndrome) disorders also were included. Patients with DKA or other concurrent acute illnesses affecting overall health and life expectancy (e.g., acute pancreatitis) were included after clinical signs of the concurrent disorder resolved and the dogs general condition had improved. Diagnostic investigations for concurrent diseases were performed at the discretion of the attending clinician. There were no dietary restrictions and, in most cases, diet was unchanged during the transition to IGla300. However, the feeding method frequently was changed from a strict 12-hourly protocol of feeding at the times of insulin injections to more frequent feeding of smaller meals or to a different twice daily feeding schedule.

Dogs only were included if their caretakers were able to perform home monitoring using the FGMS. The FGMS sensors were applied in the hospital or at home by owners. Owner perception of clinical signs and standardized assessment of FGMS data using the treatment and monitoring guidelines that were established *a priori* informed dose adjustments and final categorization into level of glycemic control. For glycemic control categorization, a 4-point scoring system was employed, synthesizing owners' perception of clinical signs into actionable categories: 1) poor/insufficient control: moderate to severe clinical signs, requiring a change in treatment; 2) moderate control: mild to moderate clinical signs, a change in treatment might be required (if undesired weight loss was a component or glycemic control was deemed insufficient for the individual case based on clinical signs, a change in treatment was required); 3) good control: mild clinical signs, and no change in treatment required; and 4) excellent control: no clinical signs, and no change in treatment required. Interstitial glucose concentration (IG) was continuously monitored using the FGMS until achievement of IG between 70-250 mg/dL (4-14 mmol/L) for >50% of the time, averaged over a 7-day period, or until glycemic control was deemed appropriate for the individual case based on clinical signs. The number of days required for dose titration was measured from the start of treatment with IGla300 to the establishment of 1 of those 2 outcomes. There was no specified maximum time for the dogs to achieve those outcomes, and dogs that remained uncontrolled throughout the study period were classified as having poor/insufficient glycemic control. Clinical hypoglycemia was defined as IG <60 mg/dL (3.3 mmol/L) associated with the presence of clinical signs (e.g., weakness, tremor, ataxia, collapse, seizures).

***A priori* guidelines for dose titration of IGla300**

1. **Initial dose**

For newly diagnosed diabetic dogs, the recommended initial IGla300 dose was 0.5 U/kg, administered SC q24h. The dose was rounded down to the nearest whole unit, and based on estimated ideal body weight rather than actual body weight in thin (body condition score [BCS] <4/9) or overweight (BCS ≥6/9) dogs. The dogs owners chose their preferred time for administering the injection.

For dogs previously treated with another insulin formulation (with the exception of insulin detemir) administered q12h, the q24h starting dose for IGla300 was calculated by adding 33% to the previous q12h insulin dose, rounded down

to the nearest whole unit. For example, if the dog was previously treated with 15U q12h, the recommended starting dose of IGla300 was 20U q24h. The first dose of IGla300 was administered 12 hours after the last dose of the previous insulin. For dogs that were already treated with another insulin formulation administered q24h, the initial IGla300 dose was the same as the dose of the previous insulin, administered at the same time of the day.

For dogs previously treated with insulin detemir, the initial q24h dose of IGla300 was calculated either by first multiplying the current q12h dose by 4 before adding 33%, or by multiplying the current q24h dose by 4.[27]

2. **Rapid dose titration based on FGMS data**

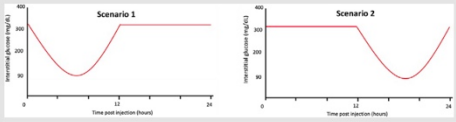
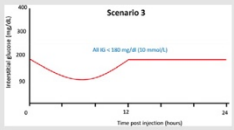
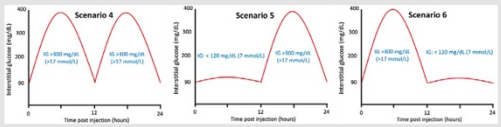
A dose increase of 10-30% (or by 1 U for dogs weighing <8 kg) was recommended every 1-3 days as long as the IG nadir was >350 mg/dL (>19 mmol/L). When the IG nadir was <350 mg/dL (<19 mmol/L), before making additional dose adjustments, it was recommended to monitor for 3-5 days or until a consistent daily pattern emerged. The guidelines in Table 1 were recommended for dose adjustments based on the observed daily pattern during this period.

3. **Indications for change to q12h dosing with or without addition of a bolus insulin injection**

An increase in dosing frequency of IGla300 was recommended (from q24h to q12h) when the IG nadir was 80-150 mg/dL (4.4-8.3 mmol/L), or nadir <80 mg/dL (<4.4 mmol/L) and mean IG >120 mg/dL (>6.7 mmol/L), and a period of approximately 12h during each 24h cycle when IG results were all >300 mg/dL (>17 mmol/L). The period of high IG could occur at any part of the 24h cycle (e.g., 0-12h or 12-24h post-injection; Table 1: scenario 1 or 2). When switching from q24h to q12h dosing, it was recommended to decrease the IGla300 dose by approximately 30% per injection (i.e., the total daily dose was increased by 40%), and to first administer the new dose 24h after the last q24h injection. After the change in frequency, the dosing decisions were to be made based on the same criteria as for q24h dosing (see previous section, "Rapid dose titration based on FGMS data"), including 3-5 days of monitoring and establishing a new pattern before re-evaluating the dose.

In diabetic dogs receiving IGla300 q24h or q12h, in which a consistent pattern of postprandial hyperglycemia emerged and control of clinical signs was not achieved, it was recommended to add a meal-time bolus injection to manage postprandial hyperglycemia. This procedure was indicated if a substantial period of IG >300 mg/dL (>17 mmol/L) consistently occurred for 4-8 hours after ≥1 meals during each 24h period (Table 1: scenario 4, 5, or 6). In these cases, Neutral Protamine Hagedorn (NPH) insulin (either as NPH or as a 70/30 NPH/regular insulin mix) or porcine lente insulin was added at ≥1 meal times at a starting dose of 0.25 U/kg, rounded down to the nearest whole unit, and based on estimated ideal body weight rather than actual body weight in thin (BCS <4/9) or overweight (BCS ≥6/9) dogs.

TABLE 1 Guidelines for insulin glargine 300 U/mL (IGla300) dose-adjustments based on flash glucose monitoring system (FGMS) data during induction of therapy.

IG nadir and/or mean	Graphic illustration of interstitial glucose pattern	Recommended dose adjustments for	
		Dogs >8 kg	Dogs <8 kg
Nadir 150-350 mg/dL (8.3-19 mmol/L)		↑10%-30% q24h	↑1 U q24h
Nadir 80-150 mg/dL (4.4-8.3 mmol/L), or nadir <80 mg/dL (<4.4 mmol/L) and mean IG >120 mg/dL (>6.6 mmol/L)		Switch to q12h dosing (with a 30% dose reduction per injection) and re-evaluate the following 3-5 days. Adjust Toujeo dose to achieve nadir between 90 and 300 mg/dL (5-17 mmol/L)	
		No change	
		Maintain IGla300 q24h and add meal-time bolus injections • Scenario 4: at hours 0 and 12 • Scenario 5: at hour 12 • Scenario 6: at hour 0 OR changing the timing and/or quantities fed at meals	
Nadir <80 mg/dL (<4.4 mmol/L) and mean IG <120 mg/dL (<6.6 mmol/L)		↓10%-30% q24h	↓1 U q24h

Abbreviations: IG, interstitial glucose concentrations.

Flash glucose monitoring system

The IG measurements were acquired with a validated FGMS (FreeStyle Libre, Abbott Laboratories Ltd, Chicago, Illinois).[18] Sensor placement was performed as previously described.[18] More than 1 generation of Freestyle Libre (i.e., Freestyle Libre 1 and Freestyle Libre 2) was used during the study period.

Statistical Analysis

Statistical analysis was performed using commercial statistical software packages (GraphPad Prism 7, San Diego, California). Descriptive statistics were generated to characterize the study population. Continuous variables were presented as median and range (minimum and maximum value). Categorical variables were described with frequencies, proportions, or percentages. Differences between dogs with or without concurrent diseases for categorical (i.e., frequency of insulin administration) and numerical variables (i.e., level of glycemic control, total insulin dose, and days to achieving glycemic control) were analyzed using the Fisher's exact test and the Mann-Whitney test, respectively. Dogs treated with topical or systemic medications that may affect glycemic control (e.g., corticosteroids, progestagens) were included in the "concurrent diseases" group. Dogs with incomplete data were excluded from the analysis. The level of significance was set at $P < .05$.

RESULTS

Study population

One-hundred and six client-owned dogs were enrolled in the study. Among these, 11 were excluded because of incomplete data (n = 5), adverse events unrelated to the treatment of DM (n = 5; including acute illness [n = 3], ophthalmic surgery complications [n = 2]), and loss to follow-up (n = 1). Of the remaining 95 dogs, 14/95 (15%) were newly diagnosed with DM, and 81/95 (85%) were previously treated with other insulin formulations. Pre-study insulins included porcine lente (40/81, 49%), NPH (20/81, 25%), IGla100 (6/81, 7%), IGla100 as basal and NPH as bolus (12/81, 15%), IGla100 as basal and porcine lente as bolus (2/81, 2%), and regular insulin (1/81, 1%). The median age was 10 years (1.5–16.1 years). There were 43 spayed females, 51 neutered males, and 1 intact male. At the time of enrollment, median body weight was 8.3 kg (1.2–35.8), and median BCS was 5/9 (1.5–8/9). Forty-two different breeds were represented. The most common breeds included mixed breed (18), Pomeranian (7), Miniature Schnauzer (7), and Miniature Poodle (6). During the study period, the type of diet remained unchanged in 83 dogs and was modified, as deemed necessary by the managing clinician, in 12 dogs.

IGla300 treatment

The median (range) IGla300 starting dose was 0.8 (0.2–2.5) U/kg q24h. At the end of the study, 56/95 (59%) dogs were receiving IGla300 q24h (median dose, 1.9 U/kg [range, 0.2-5.2]), and 39/95 (41%) dogs IGla300 q12h (1.9 U/kg/day [0.6-5.0]). Considering all dogs, the final insulin dose was 1.6 (0.2–5.2) U/kg per injection and the total daily insulin dose was 1.9 (0.2–5.2) U/kg/day. Meal-time bolus injections (30/70 regular/NPH insulin [n = 3], porcine lente insulin [n = 2]) were added in 5/95 (5%) dogs (3 dogs receiving IGla300 q24h and 2 dogs IGla300 q12h). Bolus insulin was administered q24h in 3 dogs and q12h in 2 dogs. The median bolus insulin dose was 0.5 (0.3–1.0) U/kg per injection.

Clinical outcomes

Glycemic control was classified as excellent in 62/95 (65%) dogs, good in 25/95 (26%) dogs, moderate in 7/95 (7%) dogs, and poor/insufficient in only 1 dog. The median time to achieve glycemic control was 16 (3–99) days. The time to achieve glycemic control was ≤ 30 days in 68/95 (72%) dogs. Clinical hypoglycemia was observed in 6/95 (6%) dogs. These episodes were observed in 3 dogs treated with IGla300 q24h, 2 dogs treated with IGla300 q12h, and 1 dog

receiving IGla300 q12h and porcine lente as bolus insulin q24h. Most episodes were mild, with 2 dogs (1 treated with IGla300 q24h and the other with IGla 300 q12h) developing severe signs that included seizures. No dogs developed DKA during the study period.

Dogs with concurrent diseases

One or more concurrent diseases were documented in 57/95 (60%) dogs. The most common diseases included naturally-occurring hypercortisolism (20/57, 35%), chronic gastroenteropathy (10/57, 18%), and acute or chronic pancreatitis (8/57, 14%). The most common medications administered concurrently included trilostane (17/57, 30%), maropitant citrate (13/57, 23%), topical (10/57, 18%) or systemic (6/57, 11%) glucocorticoids, pancreatic enzymes (6/57, 11%), and fenofibrate (5/57, 9%).

At the end of the study period, 29/57 (51%) dogs with concurrent diseases were receiving IGla300 q24h, and 28/57 (49%) dogs IGla300 q12h. Meal-time bolus injections (NPH insulin [n=2], porcine lente insulin [n=1]) were added in 3 dogs with concurrent diseases (2 dogs receiving IGla300 q24h and 1 dog receiving IGla300 q12h). Dogs without concurrent diseases were more likely to receive IGla300 q24h when compared with dogs with concurrent diseases (72% versus 50%, $P = .04$; Figure 1). The median total insulin dose in dogs with concurrent diseases was 2.1 (0.2–5.2) U/kg/day. No differences were observed in the median total insulin dose between dogs with and without concurrent diseases ($P = .09$, Figure 2). In dogs with concurrent diseases, glycemic control was classified as excellent in 38/57 (67%) dogs, good in 17/57 (30%) dogs, and moderate or poor/insufficient in 1 dog each. No differences were observed in levels of glycemic control between dogs with and without concurrent diseases ($P = .47$). The median time to achieve glycemic control in dogs with concurrent diseases was 17 (4–99) days. The number of days required to achieve glycemic control did not significantly differ between dogs with and without concurrent diseases ($P = .81$). Clinical hypoglycemia was observed in 4/57 (7%) dogs with concurrent diseases and in 2/38 (5%) dogs without concurrent diseases. One of the 2 dogs that experienced severe signs of hypoglycemia had concurrent diseases.

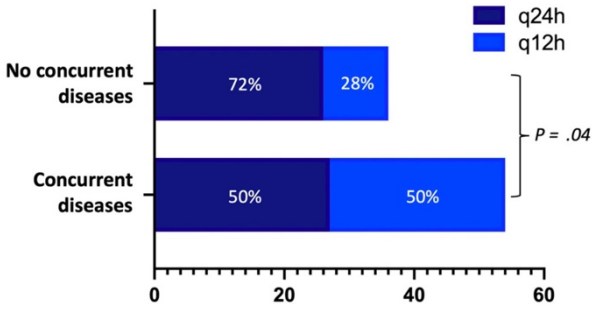


FIGURE 1 Comparison of insulin administration frequency between dogs with concurrent diseases (n = 54) and those without (n = 36). Dogs receiving meal-time bolus injections (n = 5) were excluded from the statistical analysis. Data are presented as frequencies.

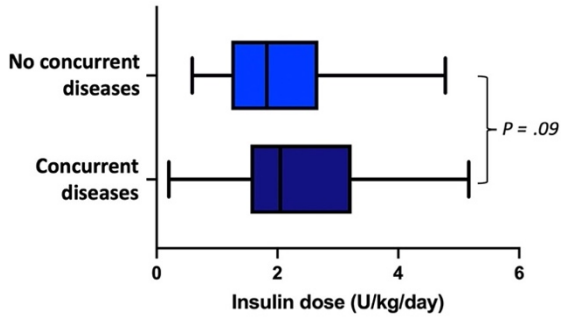


FIGURE 2 Box plots of the total insulin dose in dogs with (n = 57) or without (n = 38) concurrent diseases. The vertical bars in the middle of the box represent the median value.

DISCUSSION

Our study demonstrates the clinical utility of a protocol for dose titration of IGla300 in diabetic dogs using continuous glucose monitoring, providing a practical alternative to traditional treatment approaches involving 12-hourly injections of intermediate-acting insulin formulations combined with consistent meal feeding. The management of DM in dogs aims to resolve or improve clinical signs, minimize potential complications, and ensure a high quality of life for both the dog and the owner.[26] Given the high risk of euthanasia for diabetic dogs if the owner feels unable to cope with the requirements of treatment, maintenance of the companion animal-human bond should be prioritized when discussing

therapeutic options.[28] Six of the top 10 concerns reported by owners of diabetic dogs relate to the impact of the daily treatment schedule on their quality of life.[29] Therefore, provision of the practical alternative reported here with more flexible feeding options and once or twice daily insulin dosing will likely ease the burden for many caregivers of diabetic dogs. The safe and timely dose titration protocol using FGMS also will allow clinicians to resolve or improve the clinical signs of DM within a relatively short timeframe.

In our study, administration of IGla300 was associated with good or excellent glycemic control in most dogs, including dogs with concurrent diseases. Continuous glucose monitoring facilitated clinical and glycemic improvement within a relatively short time period, and the majority of dogs (72%) were considered clinically controlled within the first month of treatment. The median time to achieving glycemic control was only 17 days, much shorter than previously reported for other insulin preparations (and different monitoring schemes) commonly used in dogs with DM.[30-33] In a study of 53 diabetic dogs treated with porcine lente insulin and monitored with traditional BGCs, the median duration of time to achieving dose equilibration was 35 days.[30] Similar results were reported in 10 diabetic dogs treated with IGla100 and monitored weekly with BGCs, in which a median of 38 days was required to achieve stable insulin doses.[31] In diabetic dogs treated with insulin detemir and NPH, glycemic control was classified as good in 30% and 73% of cases, respectively, at the end of the 6-month (detemir) and 3-month (NPH) study periods.[32,33] In a more recent study evaluating the efficacy of protamine zinc insulin (PZI) in 276 diabetic dogs and using BGCs, glucose parameters stabilized at day 42 after treatment initiation.[34] We believe that the shorter time required to achieve glycemic control in our study was the result of using FGMS for monitoring in combination with the administration of an insulin formulation associated with relatively low day-to-day variability.[7] Additional studies will be needed to assess the relative contributions of using IGla300 versus monitoring with FGMS to the results reported here.

Dose titration for IGla300 is feasible only if patients are monitored using the FGMS, because decisions on the frequency of insulin administration and the introduction of bolus insulin can only be made using day-to-day and 24-hourly assessments of IG concentrations. The guidelines we used for dose adjustments in our study contrast with those reported for other insulin formulations.[35,36] Traditional recommendations were that insulin doses should not be adjusted more frequently than every 7 to 14 days.[35,36] In our study, dose adjustments were recommended every 1 to 3 days, likely contributing to achieving glycemic control in a shorter timeframe. Rapid dose adjustments were deemed safe because of the inherently low day-to-day variability of IGla300, and were facilitated by the relatively intensive monitoring provided by the FGMS.[7] To the best of our knowledge, ours is the first study in which insulin dose titration in client-owned diabetic dogs relied on FGMS monitoring in the home environment. Previously, serial BGCs have been the most commonly recommended method for guiding insulin dose adjustments in diabetic dogs,[35,36] These have several disadvantages, such as the need for repeated blood sampling and the risk of missing the blood glucose peak or nadir.[20] Importantly, BGCs do not allow easy assessment of glycemia on consecutive days. This concern limits their usefulness, especially for assessment of older insulin formulations that are associated with substantial day-to-day variability,[7] necessitating a more cautious approach to dose titration. In recent years, glucose monitoring has been revolutionized by the use of the FGMS.[37] The FGMS allows real-time and comprehensive assessment of glycemic excursions occurring throughout the day and night, as well as of glucose variations over consecutive days, enabling clinicians to make quicker and more informed decisions about insulin dose titration.[37] However, monitoring using FGMS is acknowledged to be relatively intensive and more costly than some simpler monitoring strategies. Consequently, some diabetic pet owners might not want to continue using the FGMS long-term.[38] After completing the dose titration period for IGla300, less intensive monitoring options often will be appropriate for ongoing long-term management of the dog's DM.

In dogs, IGla300 is reported to have lower potency compared with other insulin preparations,[17] which typically results in a lower risk of hypoglycemia and may require higher therapeutic doses. It also might provide advantages, especially in small dogs, allowing small adjustments in the effective dose with every unit change. The median dose of IGla300 to achieve glycemic control was 1.9 U/kg/day (with a range up to 5.2 U/kg/day), which is higher than doses typically required to achieve glycemic control in dogs treated with other insulin formulations.[30-34] However, the effective dose range in our study might have been over-estimated because of the presence of concurrent diseases in 60% of the dogs. Although we initiated our protocol with a low starting dose of 0.5 U/kg q24h, rapid dose titration facilitated by use of the FGMS was crucial to treatment success because it minimized the period of under-dosing in dogs that eventually required higher doses.

The option of q24h insulin administration provides substantial practical benefit for the owners of diabetic dogs because it essentially halves the potential impact on their lifestyle compared with a q12h schedule. In our study, glycemic control was achieved with q24h administration of IGla300 in 50% and 72% of the dogs with and without concurrent diseases, respectively. Insulin formulations that are most commonly used in dogs, such as porcine lente, NPH, and detemir, typically are administered q12h.[36] Recently, q24h administration was reported in 135 of 224 otherwise healthy diabetic dogs treated with PZI.[34] In that study however, only 57% of dogs were still on q24h dosing at the end of the study period,[34] compared with 72% of dogs without concurrent diseases in our study. Importantly, clinical hypoglycemia was reported in 9% and seizures in 6% of the dogs in the PZI study. It is unclear how many of these hypoglycemic events were specifically associated with q24h administration of PZI, but it is likely that the risk of hypoglycemia increases with lower frequency of administration of PZI. In comparison, only 5% of dogs without concurrent diseases in our study experienced hypoglycemia, with seizures occurring in only 1 dog. However, comparison between the 2 studies is challenging because of differences in monitoring protocols and study populations, including differences in the proportion of insulin-naive dogs (15% IGla300 versus 56% PZI) and the exclusion of dogs with concurrent disease from the PZI study (in contrast to 60% of dogs with concurrent diseases in our study). Of note, the frequency of hypoglycemia observed in our study also was much lower than that reported in dogs treated with q12h porcine lente (38.6%), insulin detemir (40%), and IGla100 (20%).[30-33] The lower rate of clinical hypoglycemia in our study could be explained by the low potency and low day-to-day variability of IGla300 and the meticulous monitoring facilitated by use of the FGMS.

The use of a basal insulin in dogs represents a paradigm shift in the overall strategy of DM treatment in dogs, because it uncouples insulin injections from feeding and provides owners with more flexibility in terms of meal timing, type of food, and consistency. In our study, the feeding method frequently was changed from a strict q12h feeding at the times of insulin injections to more frequent feeding of smaller meals or a different twice daily feeding schedule. This approach is in contrast to the traditional rigid recommendation of dividing the daily caloric intake into 2 meals that must be fed (and then consumed in full and digested) at the times of insulin injections in order to avoid hypoglycemia.[33,34] Critically, the use of a basal insulin allows for meals to be skipped without risking hypoglycemia. This approach is translated into decreased stress for owners and better control on days when meals are either deliberately withheld (e.g., in preparation for anesthesia), refused, or vomited.

The addition of bolus insulin was deemed necessary in only 5% of dogs to control substantial periods of high IG after ≥1 meals per day. This result was not surprising, considering our previous experience with a once-weekly basal insulin that was associated with good glycemic control in dogs without the addition of bolus insulin.[39] Importantly, a critical difference between DM management in dogs and people lies in treatment goals. In dogs, the main goal is clinical control, whereas in people, euglycemia is desired. Therefore, in dogs, postprandial hyperglycemia does not necessarily require treatment, unless its magnitude leads to clinical signs. If choosing to add bolus insulin, the time-action profile of

the exogenous bolus insulin should mimic physiologic bolus insulin secretion in the healthy animal. Considering physiologic bolus insulin secretion in dogs,[40-42] intermediate-acting insulin might provide a better approximation of bolus insulin secretion in many dogs than rapid-acting formulations that typically are used for this purpose in diabetic people.[2] In our study, when bolus insulin was required to manage postprandial hyperglycemia, we opted to add NPH insulin (either as NPH or as a 70/30 NPH/regular insulin mix) or porcine lente insulin once or twice daily to the treatment protocol. A starting dose of 0.25 U/kg at the time of feeding was used, and it should be noted that the final recommended doses all were higher (median, 0.5 U/kg).

Our study had several limitations, including the subjective assessment of glycemic control. We chose not to categorize glycemic control based on FGMS data because the FGMS data was used for making treatment decisions. For glycemic control categorization, a 4-point scoring system was employed instead of the ALIVE diabetic clinical score.[26] The latter, which is excellent for standardization in retrospective studies, is not useful for decision-making because it lacks a threshold for treatment. Additionally, a dog might have a low score but still require a dose increase (e.g., 3/12 score in a dog with severe polyuria and polydipsia). The 4-point scoring system enabled us to summarize the clinical assessment into a single number and allowed clinicians to assess clinical signs as they deemed appropriate. Another potential limitation is the enrollment of dogs previously treated with insulin and dogs with concurrent diseases. In this population, the positive clinical outcomes of IGla300 and the monitoring protocol we used might therefore be underestimated. The decision to include dogs already on treatment and with concurrent diseases was made in order to evaluate IGla300 in a heterogenous population that was as similar as possible to that encountered in a clinical setting, at least that of referral practices. The potential underestimation of treatment success only emphasizes the potential utility of the protocol described in our study. The use of a control group would have been advantageous as a direct comparison between IGla300 with more commonly used insulin products. However, doing so would have required resources beyond those available to us at the inception of the study. Moreover, any insulin formulation chosen as control would have represented a single option out of a large variety of insulin formulations available. Finally, the fact that all treatment and monitoring expenses were borne by the owners might have biased the study towards perceived success of the treatment.

In conclusion, basal insulin treatment of diabetic dogs with IGla300 provides a practical alternative to traditional treatment approaches using q12h injections of intermediate-acting insulin formulations and regular feeding of meals. This novel protocol represents a paradigm shift in the overall strategy of DM treatment in dogs, because it uncouples insulin injections from feeding, providing owners with more flexibility in terms of timing, type, and consistency of meals. It thus provides an opportunity to improve the quality of life and alleviate the treatment burden for many caregivers of diabetic dogs. Administration of IGla300 was associated with good or excellent glycemic control and low frequency of clinical hypoglycemia in most dogs, including dogs with concurrent diseases. Once-daily administration of IGla300 achieved good glycemic control in the majority of diabetic dogs without concurrent diseases and in half of dogs with concurrent diseases. In some dogs, q12h administration is required and in a few, the addition of meal-time bolus insulin might be necessary. Dose titration for IGla300 is only feasible if patients are initially monitored using FGMS. After completing the dose titration period, less intensive monitoring methods can be employed.

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3.3 | Insulin degludec 100 U/mL for treatment of spontaneous diabetes mellitus in dogs

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ABSTRACT

Background

Insulin degludec has several advantages including consistent release, predictable glucose lowering effect, and less day-to-day variability which is beneficial in the treatment of canine diabetes mellitus.

Hypothesis/Objectives

To describe the use of insulin degludec 100u/mL in client-owned dogs with diabetes mellitus including level of diabetic control and any adverse effects.

Animals

33 client owned dogs with diabetes, newly diagnosed (naïve) or previously insulin treated (non-naïve), with or without co morbidities and with or without concurrent medications completed the study.

Methods

A prospective, multi-institutional, uncontrolled study. Clinical signs and continuous glucose monitoring (CGM) data were monitored and guided insulin dose adjustments. A per protocol analysis was performed.

Results

The median final dose of insulin degludec in dogs with diabetes mellitus was 1.3 U/Kg (range 0.4 – 2.2) achieved in median of 14 days (range 3 – 32). Seventy nine percent of the dogs had co-morbidities with 42% having more than one co-morbidity. Sixty four percent of dogs were receiving concurrent medications with 62% receiving more than one non-insulin medication. Seventy-six percent of dogs were scored as having excellent/very good diabetic control. Dogs showed significant improvements in both ALIVE diabetic clinical score ($p=0.0007$) and average 3-day interstitial glucose ($p<0.0001$) from baseline to study exit.

Conclusions and clinical importance

Insulin degludec 100 U/mL is effective for treatment of dogs with diabetes mellitus. Eighty-four percent of dogs responded with once daily dose of insulin degludec with low frequency of clinical hypoglycemia.

INTRODUCTION

Insulin degludec (IDeg) differs from human insulin in that replacing the B30 amino acid is fatty acid (hexadecanoic acid) that is linked to lysine at B29. Formulated with phenol and zinc, IDeg forms strand-shaped multihexamers in subcutaneous depot that gradually release monomers into circulation as zinc diffuses out of the multimer. Insulin degludec binds to albumin and dissociates slowly prior to insulin receptor binding. In people, the half-life of IDeg is >40 hours and it mimics basal insulin secretion with consistent release and minimal intraday and inter-day variability.[1,2] In healthy dogs, duration of action lasts more than 20 hours with a flat time action profile.[3] In an induced-diabetes canine model, IDeg administered twice daily was associated with less intra-day variability when compared to a Lente insulin.[4] Reported benefits of IDeg in people include more predictable glucose lowering effect allowing more rapid dose escalation, tighter glycemic control and less risk of hypoglycemia compared to insulin glargine 300 U/mL (IGla300).[1] The administration of the basal insulin, IGla300, in dogs with DM results in good or excellent glycemic control with a low incidence of hypoglycemia.[5]

In the fall of 2022, Novo Nordisk released an unbranded biologic of Tresiba® (IDeg) at 65% price reduction in the US market. By spring of 2023, IDeg was readily available and for the first time economically feasible for treatment of dogs with diabetes mellitus (DM) in the US. With the discontinuation of insulin detemir (Levemir®) in 2024, there is a compelling and timely need for investigation of alternative insulins for treatment of canine diabetes mellitus (DM).

Our objective in this study was to describe insulin degludec 100 U/mL (IDeg100) in client-owned dogs with DM that are either newly diagnosed ("naïve") or transitioned from another insulin formulation ("non-naïve").

MATERIAL AND METHODS

2.1 Study design and population

This prospective, multi-institutional, uncontrolled study evaluated IDeg100 in client-owned dogs. Inclusion was considered for any dog with DM, whether the dog was "non-naïve" or "naïve" to insulin treatment. Dogs were included regardless of prior duration of DM and whether comorbidities were present or concurrent medication were administered. Diabetes mellitus was diagnosed according to criteria established by Agreeing Language in Veterinary Endocrinology (ALIVE) project for the "naïve" group.[6] Dogs were excluded if they would not tolerate a flash glucose monitoring system (FGMS) or continuous glucose monitoring system (CGM) for the study duration or if the pet owner was not willing to participate. The study population was drawn from 4 referral centers from February 2023 to April 2024. This trial was approved by Institutional Animal Care and Use Committee of the University of Florida. This study was internally funded.

2.2 Data collection

Guidelines for data collection were established a priori. At the time of enrollment, data collected included age, weight, body condition score (BCS), sex and neuter status, breed, Agreeing Language in Veterinary Endocrinology (ALIVE) diabetic clinical score (DCS) [6], presence of co-morbidities and concurrent medication administration. For "non-naïve" dogs, dose and type of insulin prior to enrollment and baseline IG average (3-day period immediately prior to enrollment) were also recorded. "Non-naïve" dogs without IG data for the 3 days immediately before enrolment were excluded from analysis of IG data. For "naïve" dogs, baseline IG average was considered as the first 3 days on IDeg100 therapy. "Naïve" dogs included dogs with newly diagnosed DM that were either otherwise systemically healthy and those with diabetic ketoacidosis (DKA). Dogs with DKA were hospitalized and treated with CRI of regular insulin prior to study enrollment. On the day of hospital discharge, IDeg100 therapy was initiated, and Freestyle libre (FSL) data (FSLD) were collected for 3 days starting on day of discharge. For all dogs, the starting dose and frequency of IDeg100, were recorded at enrollment. A dog completed the study when the clinician judged the dog to have reached final IDeg100 dose, as determined by the combination of clinical signs, DCS, and FSLD. Diabetic control (DC) was scored at the end of the

study and was categorized as follows 1. Poor control: severe clinical signs necessitating treatment change; 2. Moderate control: moderate clinical signs that may require treatment change; 3. Good control: minimal clinical signs with no change in treatment required; 4. Very good to excellent control: no clinical signs with no required change in treatment. At study exit, the following were also recorded: DCS, final dose and frequency of insulin, 3-day average IG on final dose, days to achieve final dose, clinical hypoglycemic episodes, diet changes and adverse effects. Clinical hypoglycemia was defined as the presence of consistent clinical signs such as lethargy, disorientation, weakness, tremors, ataxia and/or seizures with documentation of corresponding IG < 60 mg/dL and positive response to feeding or oral or IV glucose administration. Pet owners were not required to document blood glucose values during the episodes. Other than insulin adjustments, any other medication changes from baseline were not recorded.

2.3 Flash or continuous glucose monitoring

Interstitial glucose measurements were obtained by FGMS (Freestyle Libre 2 or Freestyle libre 14-day) or CGM (Freestyle Libre 3) based on owners' preference. Freestyle libre 14-day system (FreeStyle Libre, Abbott Laboratories, Chicago, IL) has been previously validated for IG measurement in dogs with DM.[7] Sensor placement was performed as previously described although location was at clinician's discretion.[7] There was no minimum requirement for length of time monitored. Rather, CGM was used as long as necessary to modify insulin therapy.

2.4 Protocol for treatment with IDeg100

2.4.1 Initial dose and administration

An IDeg100 insulin pen was used by pet owners for administration in all dogs. The insulin pen was removed from the refrigerator and allowed to warm to room temperature over 10 minutes. This protocol was repeated prior to each insulin injection. The pet owner then removed the cap from the insulin pen and added a new needle before each injection. The required IDeg100 units were then dialed on the pen. The pet owner pinched the skin at the chosen site, held the pen like a writing pen, inserted the pen into the skin at 45 degrees and pushed the button. The site for insulin injection was at owner's discretion and not recorded. The insulin pen was removed as soon as the dial returned to "zero" and placed back in the refrigerator.

In "naïve" dogs, the recommended initial IDeg100 dose was 0.5 U/Kg based on actual body rounded down to the nearest whole unit, administered subcutaneously q24h. The dog's owners chose their preferred time for administering the injection.

In "non-naïve" dogs treated with NPH and porcine lente insulin administered q12h, the q24h starting dose for IDeg100 was calculated by adding 30% to the previous q12h insulin dose, rounded down to the nearest whole unit. For example, if the dog was previously treated with 10U q12h, the recommended starting dose of IDeg100 was 13U q24h. The first dose of IDeg100 was administered 12 h after the last dose of the previous insulin. For dogs that were treated with porcine lente or NPH insulin q24h, the initial IDeg100 dose was 30% lower than the dose of the previous insulin administered at the same time of day. Dogs previously treated with IGla300 q24h were transitioned to the same dose of IDeg100. There were no dogs previously treated with Igla300 q12h. There were no requirements for feeding frequency or timing of feedings in relation to insulin administration and was determined solely by owner preference.

2.4.2 Rapid dose titration based on FGMS data

Dose titration followed the protocol previously published for IGla300.[5] In brief, a dose increase of 10-30% (or by 1 U for dogs weighing less than 8 kg) was recommended every 1-3 days as long as the IG nadir was >350 mg/dL (>19 mmol/L). When the IG nadir was <350 mg/dL (<19 mmol/L), prior to making additional dose adjustments, it was recommended to monitor for 2-3 days or until a consistent daily pattern emerged. A consistent daily pattern of IG on

FSLD refers to the trend of glucose readings over a 24-hour period compared to previous 24-hour periods and includes consistent time and magnitude of nadirs and post prandial peaks (Figure 1). This is a subjective assessment. An increase in dosing frequency from q24h to q12h was recommended when the IG nadir was <150 mg/dL (4.4-8.3 mmol/L) and IG results were all >300 mg/dL (>17 mmol/L) for 12h or greater duration during each 24h cycle. When switching from q24h to q12h dosing, it was recommended to decrease IDeg100 by about 30% per injection (i.e., the total daily dose was increased by 40%), and to first administer the new dose 12h after the last q24h injection. After the change in frequency, the dosing decisions were to be made based on the same criteria as for q24h dosing.

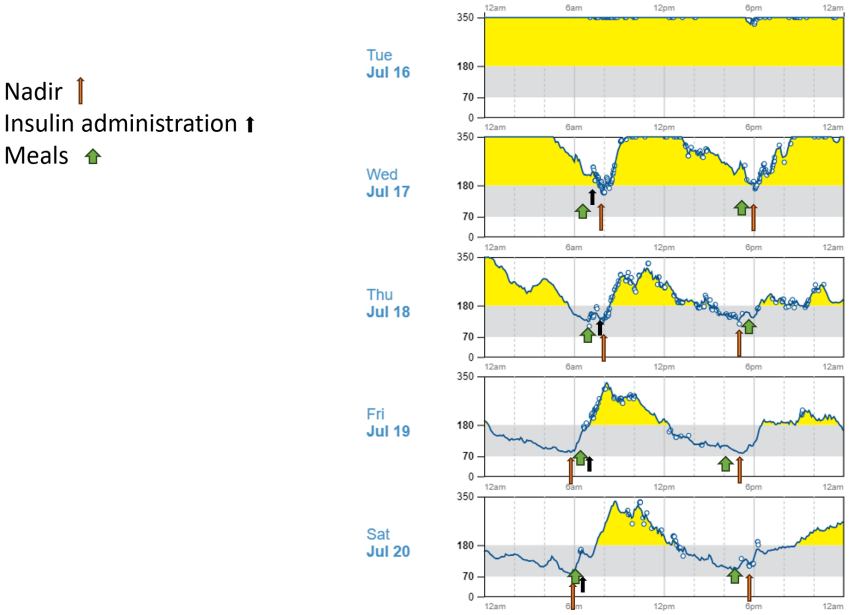

Figure 1: Example of consistent IG pattern in dog receiving IDeg100. On July 16, the dog received an IDeg100 dose that increased by 20% from the day before. The dog starts to exhibit a consistent response to IDeg100 on July 17. Over the next few the time and magnitude of the nadir and post prandial peaks become becomes more consistent with each day. There was no adjustment in insulin dose after July 16. Time of insulin administration is represented by black arrows, time of meals by green arrows and IG nadirs by red arrows.

In diabetic dogs receiving IDeg100 q24h or q12h, in which a consistent pattern of postprandial hyperglycemia emerged, and control of clinical signs was not achieved, it was recommended to add a meal-time bolus injection of Neutral Protamine Hagedorn (NPH) or a 70/30 NPH/regular insulin mix at a starting dose of 0.25 U/Kg. The bolus insulin dose was based on actual body weight rounded down to the nearest whole unit.

Clinicians' decisions on rapid insulin dose titration were based on the above protocol and daily FGMS/CGM data with daily owner emails and communications. Owners were asked to send daily email updates to the clinician informing of dose and timing of insulin injections, feeding times, amounts and diet type, timing of exercise, changes in behavior, urination or thirst, clinical hypoglycemic episodes and any stressors.

The final insulin dose and frequency were at discretion of clinician, guided by clinical signs, FSLD , and DCS, with the goal of achieving the lowest clinical score while minimizing the risk of hypoglycemia . Diet changes (including type, frequency, and meal proportions) were permitted based on the discretion of the attending clinician and owner preferences. The study was concluded for an individual dog when final IDeg100 dose was achieved, or when diabetic control could not be achieved on IDeg100 and the dog was switched to another insulin formulation, or when the dog was lost to follow up, owners withdrew consent, or when the dog died.

2.5 Statistical analysis:

Statistical analysis was performed using commercially available computer software (GraphPad Prism version 10.0.2; GraphPad Software LLC, CA, USA). The analysis was restricted to dogs that completed the study per protocol rather than intent to treat analysis. Descriptive statistics were applied to the study population. Categorical data was expressed as percentages within the affected population. Numerical data was assessed for normality by Shapiro-Wilk test. Data that was not normally distributed were reported as median and range and normally distributed data as mean ± standard deviation. For baseline and post DCS and average 3-day IG, data was analyzed in 3 groups – "all dogs", "naïve" and "non-naive". Baseline and post DCS and baseline and post average 3-day IG in "naïve" dogs were investigated with non-parametric testing (Wilcoxon test). Baseline and post average 3-day IG in "all" and "non-naive" dogs were analyzed with paired t-tests . Sample size calculation and power analysis were based on the expectation that 3-day average IG at study exit will be lower than 3-day average at baseline by at least 100 mg/dL. Assuming an SD of 150 mg/dL, for 80% power and alpha = 0.05, a minimum of 21 dogs was necessary (https://statulator.com/SampleSize/ss2PM.html).

RESULTS

3.1 Study population

Forty-nine client owned dogs were enrolled. Of these, 16 were excluded from final analysis because they did not follow study protocol to completion. (Figure 2). Of these 16, 10 were lost to follow up or had incomplete data (In 2 there was no further contact and in 8 there was minimal participation beyond the initial screening visit). Four of the 16 dogs were excluded because owners chose to switch to another insulin for reasons unrelated to the study protocol. Of these 4, 2 dogs exited after 7 and 14 days to join another clinical trial that investigated another insulin and one dog switched back to its previous insulin after 48 hours of IDeg100 due to increased polyuria and polydipsia. The fourth dog (55.3 kg) switched back to previous insulin after 16 days of IDeg100 due to owner's anticipated financial concerns regarding their ability to afford IDeg100 long-term. Two of the 16 dogs had fatal outcomes (1 euthanasia, 1 died). One dog had been hospitalized for DKA and acute kidney injury and was euthanized at the primacy care veterinarian 9 days later for declining health. The other dog died of unknown causes 6 days after starting IDeg100. The owner did not seek subsequent veterinary care nor advice prior to the dog's demise. Necropsy was not performed on either dog.

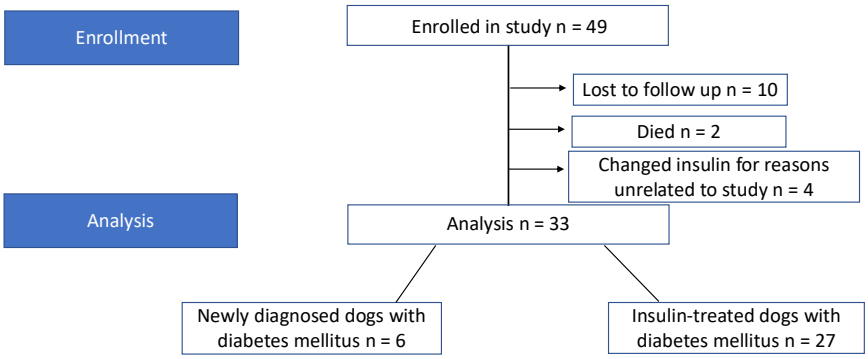

Figure 2: Flow diagram of enrollment of treatment of dogs with diabetes mellitus with insulin degludec

Thirty-three dogs completed the study among which there were nineteen males (17 neutered; 2 intact) and fourteen females (14 spayed). Most common breeds were mixed breed (n=17), Yorkshire terrier (n=2), pug (n=2) and shih tzu (n=2) with an additional 10 dogs of 10 different breeds. The median age was 11.2 years (4.4 – 15.5). At enrollment, the median weight was 9.1 kg (3.6 – 52.6) and median body condition score was 6/9 (4 – 9) with a non-normal distribution. Eighty two percent of dogs (27/33) were transitioned from another insulin including porcine lente (19/27,

70%), neutral protamine Hagedorn (NPH) (4/27, 15%), IGla300 (2/27, 7%), porcine lente with IGla300 (1/27, 4%), and NPH with IGla300 (1/27, 4%). Eighty five percent of those dogs (23/27) had 3-day IG data immediately prior to enrollment. Whereas fifteen percent (4/27) did not have pre-enrollment 3-day IG data on previous insulin and thus were not included in analysis of IG data. Eighteen percent (6/33) of dogs were newly diagnosed ("naïve"), with 33% (2/6) of those presenting in DKA. Seventy-nine percent of the dogs had co-morbidities (26/33) as described in Table 1. Forty-two percent (11/26) had more than one co-morbidity. All "naïve" dogs with DM (6/6) had co-morbidities whereas 74% of "non-naïve" dogs (20/27) had co-morbidities. Sixty-four percent (21/33) were receiving medications together with insulin as summarized in Table 2. Sixty-two percent (13/21) were receiving more than one non-insulin medication with 29% (6/21) of those receiving medications in more than one of the medication categories. Eighty three percent (5/6) of "naïve" dogs with DM had concurrent medications compared to 63% (17/27) of "non-naïve" dogs. Twenty-seven percent (9/33) of dogs had a change in diet, change in proportion of canned and dry diet fed or amount fed per meal at the start of the study. Of these, 44% (4/9) transitioned to a low-fat diet, 11% (1/9) to a prescription diet for DM, 11% (1/9) to a weight loss diet, 11% (1/9) to a maintenance diet, 11% (1/9) to less kibble and more canned of the same diet and 11% (1/9) changed from 2 equal meals to 65% in the morning and 35% in the evenings.

Co-morbidity category (total 26 dogs)	Number of dogs affected (%)
Ophthalmic diseases including cataracts	8 (31)
Pancreatitis	5 (19)
Hypertriglyceridemia	4 (15)
Heart disease	3 (12)
Urinary tract infection	3 (12)
Suspect hyperadrenocorticism	2 (8)
Adrenal tumor	2 (8)
Intervertebral disc disease	2 (8)
Mass – spleen 1/submandibular 1	2 (8)
Hypothyroidism	1 (4)
Epilepsy	1 (4)
Calcium urolithiasis	1 (4)
Presumptive gastrointestinal ulceration	1 (4)
Immune mediated hemolytic anemia	1 (4)
Chronic enteropathy	1 (4)
Chronic renal disease	1 (4)
Hypertension	1 (4)
Tracheomalacia	1 (4)

Table 1: Categories of co-morbidities in dogs with diabetes mellitus

Concurrent medication category (total 21 dogs)	Number of dogs (%)
Ophthalmic	7 (33)
Gastrointestinal related	7 (33)
Arthritis or pain related	4 (19)
Anti-lipidemic	3 (14)
Cardiac	2 (10)
Appetite stimulants	2 (10)
Dermatologic	2 (10)
Antibiotic	1 (5)
Hypertensive	1 (5)
Thyroid	1 (5)
Anti-tussive	1 (5)
Immune suppressive	1 (5)
Anti-thrombotic	1 (5)

Table 2: Categories of concurrent medications in dogs with diabetes mellitus

3.2 Characteristics of IDeg100 treatments

In all dogs, IDeg100 was initiated at once-a-day frequency with a median dose of 0.6 U/Kg (0.3 – 1.4). Eighty five percent (28/33) of dogs were maintained on once-a-day frequency throughout the study. Of those, one dog required the addition of a twice daily bolus insulin (NPH) . Of dogs receiving once-daily IDeg100, 57% (16/28) received the injection in the morning compared to 43% (12/28) in the evening. Fifteen percent (5/33) of dogs were transitioned to twice daily dosing and none required the addition of a bolus insulin. Of those 5, 80% (4/5) had co-morbidities and 60% (3/5) received concurrent medications. Of the dogs that completed the study on once daily insulin, seventy nine percent (22/28) had co-morbidities and 68% (19/28) received concurrent medications. The final total daily dose of IDeg100 in all dogs was 1.3 U/Kg/day (0.4 – 2.2), reached in 14 days (3 – 32). Ten percent of dogs (3/33) reached final IDeg100 dose in 3-4 days and 27% (9/33) within 7 days. In "non-naïve" dogs, the total daily of the previous insulin formulation dose was 1.4 U/Kg/day (0.5 – 2.7) compared to a final IDeg100 dose of 1.4 U/Kg/day (0.6 – 2.2, p=0.5). To determine the theoretical risk of hypoglycemia with IDeg100 dosing strategy used in the study (i.e. a starting dose of 30% greater than the previous suspension insulin not including Igla300), the final IDeg100 injection dose (and considering the higher dose if AM and PM differed), whether once or twice daily, was compared to previously administered suspension insulin (n=23,

considering the lower dose if AM and PM differed). In 2 of 23 cases (9%) the IDeg100 injection dose was lower than the original suspension dose (by 23% and 30%), in 1 dog it was the same and in a fourth dog it was higher than the original suspension dose by only 12%. In all 4 dogs, therefore, the IDeg100 dose had to be decreased from the original starting dose. None of these 4 dogs experienced clinical hypoglycemia.

The final total daily dose of IDeg100 in dogs with or without co-morbidities was 1.3 U/Kg (0.4 – 2.2) and 1.4 U/Kg (0.8 – 1.9) respectively (p=1.0). Dogs not receiving concurrent medications had a final total IDeg100 dose of 1.1 U/Kg (0.8 – 1.9), similar to dogs receiving concurrent medications (1.4 U/Kg [0.4 – 2.2], p=0.4). One dog with co-morbidities on once daily IDeg100 at initial dose of 0.8 ug/kg experienced clinical hypoglycemia with seizures that required treatment. The dose of IDeg100 was decreased to 0.4 u/kg and the dog completed the study. In this dog, IG values < 60 mg/dL were documented in 4.3% (25/576) of readings over 2 days circa the hypoglycemic event. Four other dogs had IG readings < 60 mg/dL during the study, but none had clinical hypoglycemia. Two of these dogs had 1.4% (4/288) and 2.4% (7/288) IG values < 60mg/dL over one day. Interstitial glucoses < 60 mg/dL were documented in 4.5% (52/1152) of readings over 4 days and 2.4% (43/1728) of readings over 6 days in the other 2 dogs. No other adverse effects were reported.

3.3 Glycemic outcomes

In dogs that completed the study protocol, clinicians' scores of DC at the final IDeg100 dose was excellent/very good in 76% (25/33) and good in 24% (8/33). No dog was scored as having moderate or poor DC. In all dogs, DCS decreased from 3 (0 – 8, 96.49% CI [2,5]) at baseline to 1 (0 – 7, 96.49% CI [1,2]) at study exit (p=0.0007;Figure 3a). Diabetic clinical scores were not normally distributed and decreased from a baseline of 3 (0 – 8, 98.08% CI [2,5]) in "non-naïve" (Figure 3b) and 5.5 (0 – 8, 96.88% CI [0,8]) in "naïve" dogs (Figure 3c) to 1 (0 – 7, p=0.0055, 98.08% CI [1,3]) and 2 (0 – 6; p=0.0938, 96.88% CI [0,6]) respectively at study exit.

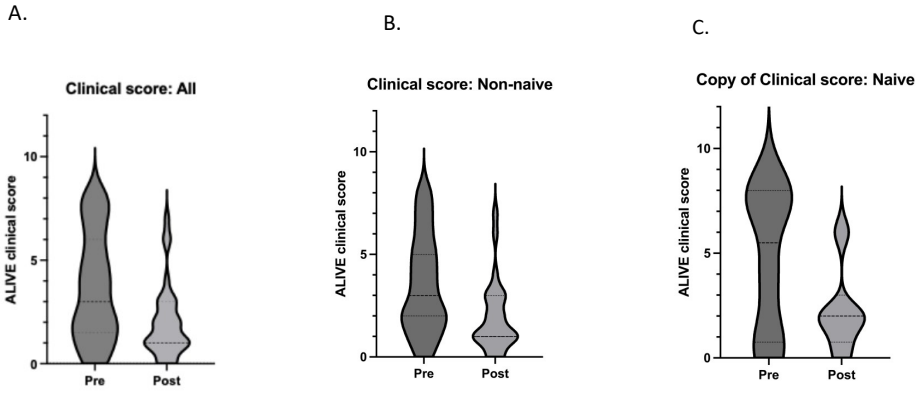

Figure 3: Violin plots of pre and post ALIVE diabetic clinical score in all (a), non-naïve (b) and naïve (c) dogs with diabetes mellitus treated with insulin degludec U100. The upper and lower dashed lines represent upper and lower confidence limits. The middle dashed line represents the median score. (p=0.0007).

In all dogs data was normally distributed and 3-day average IG decreased from 332.8 ± 68.7 mg/dL at baseline to 229.0 ± 56.3 mg/dL (p<0.0001) at study exit (Figure 4). Data was normally distributed in "non-naïve" dogs, and the 3-day average IG decreased from 340.1 ± 66.4 mg/dL at baseline to 230.5 ± 55.35 mg/dL (p<0.0001) at study exit (Figure 5). Data in "naïve" dogs was not normally distributed, and the 3-day average IG decreased from 328.3 mg/dL (164.1 – 403.1, 96.88% CI [200,389.6]) at baseline to 195.6 mg/dL (163.6 – 258.9; 96.88% CI [160.9,240.1],p=0.1) at study exit. Comparing dogs with or without co-morbidities at study exit, there was no difference in DCS (1.5[0 – 7 vs. 1 [0 – 3]

respectively; p=0.4), 3-day average IG (232.6 mg/dL [126.3 – 333.5] vs. 217.8 [129.3 – 376.6] respectively, p=0.6), or DC (excellent/very good in 86% [6/7] and 70% [18/26] respectively, p=0.6).

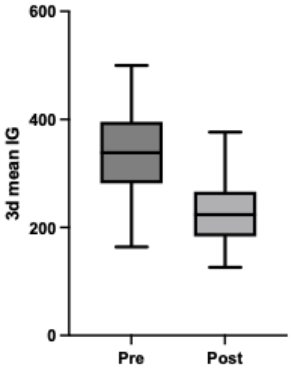

Figure 4: Mean 3-day average interstitial glucose at baseline and after treatment with insulin degludec 100 in dogs with diabetes mellitus. Legend: The box and whiskers plots represent average mean 3-day interstitial glucose at baseline and after treatment with insulin degludec 100 in dogs with diabetes. The lower and upper lines of the box represent upper and lower 95% confidence interval of mean. The middle line represenets the mean average 3-day interstitial glucose (p<0.0001).

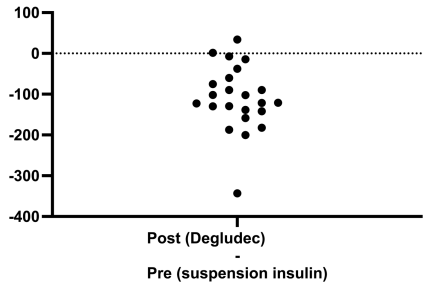

Figure 5: 3-day mean IG (mg/dL) post degludec insulin therapy in non-naïve dogs treated with suspension insulin. The circles represent individual dogs. Zero represents no change in in post 3 day average IG. Values below zero indicate there was a decrease in 3-day average IG after degludec therapy.

DISCUSSION

Dogs with DM, both "naïve" (newly diagnosed) and "non-naïve"(those transitioned from another insulin formulation) treated with IDeg100 showed improvement in DCS and 3-day average IG over the course of the study. All dogs were considered to have good to very good/ excellent DC on IDeg100, most on once daily administration, with only a single dog requiring the addition of a bolus insulin to achieve good control. Presence of co-morbidities or concurrent medications was common in this group of dogs with DM. There was one episode of clinical hypoglycemia which required intervention.

Basal insulins have previously been shown to be effective in treatment of dogs with DM.[5,8] Insulin degludec 100U/mL offers less day-to-day variability in dogs with DM compared to porcine lente .[4] This increased predictability of IDeg100 allows for rapid insulin dose titration with FGMS or CGM as described with IGla300.[5] Administration of a basal insulin does not require strictly timed q12h equally proportioned meals which lessens caregiver burden by allowing owners more schedule flexibility. Previously, the use of the basal insulin IGla300 was associated with lower risk of hypoglycemia compared to traditional intermediate-acting insulin suspensions.[5,9] In the study reported here, the frequency of clinical hypoglycemia with IDeg100 was similar to IGla300 (3% vs. 6% respectively). Future randomized controlled clinical trials that compare these 2 formulations side by side are needed to determine if there is any significant difference in risk of clinical hypoglycemia.

A median dose of 0.6 U/Kg of IDeg100 was initiated and dose escalated to a median total final IDeg100 dose of 1.3 U/Kg over median of 14 days. The median time to achieving glycemic control compares favorably to other insulin formulations such as IGla300 (17 days), porcine lente (35 days), IGla100 (38 days), and protamine zinc insulin (42 days).[10–12] The shorter time period for dogs to achieve adequate glycemic control is likely due to a combination of the consistency and predictability of IDeg100 and the use of FGMS or CGM monitoring allowing rapid dose escalation. Of course, in most of these studies, more variables differed from our study (including population studied, investigator, etc.) and it is impossible to isolate this difference to an exclusive effect of insulin type. A more direct comparison can be made with the results of the study on IGla300 which used the same dose escalation protocol. Whether the small difference in

time to achieving control between these 2 studies is the result of the difference in insulin formulations or some other random effect can only be determined by future randomized control clinical trials that compare these 2 formulations side by side. While the protocols were identical in these 2 studies, clinicians were not. Also, in both studies, the dose escalation protocol was meant to serve as guideline only, with final decisions left to the discretion of the attending clinicians. As such, experience gained with IGla300 in the Tardo et al. 2024 study might have affected our use of the dose-escalation protocol and possibly led to even more rapid dose escalation, as confidence of clinicians with the use of a basal insulin grew. Like the IGla300 study, our study did not set a rigid timeline for rechecks or for dose-escalation decisions based on CGM and we did not have built-in incentives for owners to maintain CGM sensors on their dogs or to report clinical data to us. As such, in several cases, inconsistent updates and loss or malfunction of sensors delayed clinicians' ability to adjust doses and achieve glycemic control more rapidly. Thus, median time to achieve DC would likely have been shorter with better owner compliance.

In "non naïve" dogs as a group, the final insulin dose was not different as the insulin dose of the previous insulin formulation. While potency cannot be evaluated in our study, especially considering the frequency of diet change, these results might suggest that overall, the potency of IDeg100 in dogs is similar or increased (considering the improved glycemic control) compared to insulin suspensions that are traditionally used in dogs. However, our protocol for IDeg100 starting dose (adding 30% to the previous q12h insulin dose) resulted in the need to decrease the dose in 4 out of 23 dogs and in 2 of those, the IDeg100 injection dose was in fact lower than their pre-study insulin suspension dose. Although none of these dogs experienced clinical hypoglycemia, this highlights the need for a more conservative approach to transitioning from a suspension insulin formulation with the expectation that the dose will likely require rapid escalation in most dogs. Our current recommendation when transitioning from q12h suspension insulin to IDeg100 q24h is to start at the previous q12h dose and not increase by 30%.

All glycemic parameters improved in IDeg100 treated dogs. This was unexpected because a major impetus to switching dogs to IDeg100 in this study was its low cost and not necessarily inadequate control on previous insulin formulations. To rule out the possibility that this result was skewed by improvement in only the "naïve" dogs, DCS and median final 3-day average IG were further subdivided into "naïve" and "non-naive" dogs. Analysis of the "non-naive" group showed significant improvement in glycemic parameters, suggesting that the basal insulin IDeg100 may be superior to traditional insulin suspensions. This result is especially important considering that most dogs on IDeg100 were treated once daily (as opposed to twice daily on suspension insulin).

The lack of significant improvement in glycemic parameters in the "naïve" dogs is likely a type 2 error related to the small sample size of this sub-group and the fact that its baseline 3-day IG average was actually measured during the first 3 days of IDeg100 treatment. Because these dogs were newly diagnosed, "pre-treatment" IG measurements were not available for them. While this was a necessary compromise, it should be noted that rapid dose escalation as described in our study likely resulted in significant improvement in glycemic control already within the first 3 days, making it less likely to observe a significant difference between the first 3 days and study exit. Indeed, rapid dose escalation in our study resulted in reaching final IDeg100 dose in as little as 3-4 days in 10% of dogs and under a week in 27% of dogs.

As previously described, co-morbidities and administration of concurrent medications are common in dogs with DM.[5,13] Over three quarters of the dogs had co-morbidities and 42% had more than one co-morbidity. The presence of one or more co-morbidities did not influence the frequency of insulin administration , the final total insulin dose , the final DCS , the final 3-day average IG or level of DC . This was an unexpected finding for two reasons. First, co-morbidities such as hyperadrenocorticism contribute to insulin resistance and increase insulin requirements.[14] Co-morbidities were common in these dogs; however, many of the dogs had local disease (like ophthalmic or lower urinary

tract disease), easily controlled systemic diseases (like hyperlipidemia, hypothyroidism) or otherwise subclinical diseases (like nonfunctional adrenal tumors or splenic masses) that may not have impaired insulin sensitivity. Secondly, in the authors' opinion, clinicians often choose not to target tight glycemic control in dogs with co-morbidities such as mature cataracts or other diseases that may influence factors such as appetite, thirst, urination and life expectancy.

As this study occurred in client-owned dogs, clinicians were free to manage these cases according to the "best" interest of the dog. Dietary and medication changes were uncontrolled variables and allowed when deemed medically necessary by treating clinicians for management of DM or co-morbidities. Some of these dietary adjustments and medication changes may have positively influenced glycemic responses to IDeg100. However, none of the dogs received anti-diabetic drugs in addition to insulin therapy. Future studies that control concurrent medications and dietary changes would be necessary to determine if these variables impact the management of DM in dogs with IDeg100.

One third of dogs did not complete the study. Of those, twenty percent (10/49) were lost to follow up or had incomplete data underscoring the challenges faced in maintaining participant engagement throughout clinical trials. This was not unexpected considering the study required a high level of owner participation and compliance but provided no incentives to owners. Owner compliance and engagement may have been improved by requiring several in-person rechecks throughout the study. However, in an effort to limit expenses for owners, no such rechecks were required. One limitation of this study was that outcome analysis was not based on intention-to-treat but rather it was performed only on dogs that completed the protocol. This might have favorably biased our results as some owners might have decided not to continue in the study because of dissatisfaction with the performance of the study insulin.

This study had several limitations. Several subgroups had a relatively small number of dogs which might have limited our ability to find differences. Several of the "non-naïve" dogs had excellent DC prior to entering the study which may have limited changes in DCS and mean 3-day average IG. The dogs with DM were managed by several clinicians at several institutions. Although all cases were overseen by a board-certified internist, differences in individual bias, judgement and management may have increased variability. In an effort to minimize some of the inter-clinician variability in decision making, two of the investigators were consulted daily either in-person or remotely for input on libre data and dose adjustments. Three models of FGMS and CGM were employed in the study of which only the FSL 14-day has been validated for use in dogs. Owner's preference determined the FSL model used. Both the FSL 2 and FSL 3 utilize the same algorithm so a significant difference between the sensors that would influence clinical decisions was not expected. Each individual dog used the same version of FSL throughout the study and was compared to its own libre results. Freestyle libre 2 and 3 do not report IG above 400 mg/dL. Thus, the actual IG values may have been underestimated in some dogs resulting in less improvement between baseline and post study values.

In conclusion, IDeg100 provides a cost-effective alternative to other standard and basal insulin therapies for canine DM in USA. The majority of dogs were adequately controlled on once daily IDeg100 , regardless of previous insulin treatment status, co-morbidities, or use of concurrent medications. IDeg100 has a low frequency of clinical hypoglycemia. Additional bolus insulin administration is rarely required to achieve adequate glycemic control. The once daily administration, short time to achieve final insulin dose, and decrease in caregiver burden, all make IDeg100 an excellent choice for treatment of dogs with DM.

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3.4 | Transmucosal glucagon rapidly increases blood glucose concentration in healthy cats

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ABSTRACT

Objectives

To evaluate the effect of transmucosal glucagon powder (Baqsimi; Amphastar Pharmaceuticals, Inc) on blood glucose (BG) concentrations in healthy cats and describe adverse reactions to its administration.

Methods

A randomized, controlled, crossover study was conducted on six healthy cats with a 7-day washout period. Transmucosal glucagon powder was administered intranasally and rectally and compared with intranasal placebo. Blood was collected at −15 and −1 mins before glucagon administration and 5, 15, 25, 35, 45 and 60 mins after to evaluate BG, plasma glucagon concentrations (pGlucagon) and plasma potassium concentrations (K+). Stress scores and adverse effects were recorded at all time points.

Results

Median pGlucagon in the nasal and rectal groups increased from baseline (nasal: 12.2mmol/l, range 3.5–44.1; rectal: 6.9mmol/l, range 2.9–21.1) to 218.5mmol/l (range 7.9–349.8; P=0.02) and 349.8mmol/l (range 67.4–349.8; P=0.01), respectively, 15mins after administration. Median BG increased from baseline (101mg/dl, range 91–110) 15mins after nasal (137.5mg/dl, range 104–251; P=0.006) and rectal (229mg/dl, range 99–285; P = 0.002) administration. Median K+ decreased from baseline (nasal: 3.8 mmol/l, range 3.6–4.1; rectal: 3.7 mmol/l, range 3.5–3.9) to 3.4 mmol/l (range 3.1–3.6; P = 0.04) at 15 mins with nasal administration, and to 3.2 mmol/l (range 3.1–3.6; P=0.04) at 15mins and 3.1mmol/l (range 2.9–3.4; P=0.01) at 25mins with rectal administration. No significant changes were detected in the placebo group. No serious adverse effects were noted.

Conclusions and relevance

Transmucosal glucagon administration is effective in raising BG with minimal side effects in healthy cats. Future studies are needed to quantify the efficacy and safety of transmucosal glucagon in diabetic cats, especially during hypoglycemic crises.

INTRODUCTION

Hypoglycemia is a major limiting factor in the management of diabetes mellitus (DM) in patients receiving insulin therapy. Diabetic humans and animals have impaired counter regulatory responses to insulin-induced hypoglycemia (IIH), which leads to a lack of, or insufficient, glucagon secretion in response to hypoglycemic events (but does not limit response to exogenous glucagon).[1-3] In people and in dogs, the use of continuous glucose monitoring (CGM) allows for a more accurate identification of low glucose episodes compared with intermittent monitoring.[4] In cats, there are currently no reports on the frequency of hypoglycemia using CGM. When home blood glucose (BG) monitoring was used with an intensive insulin protocol, a high frequency (94%) of subclinical hypoglycemia (BG <50mg/dl) was reported, with low frequency (2%) of clinical hypoglycemia.[5] While the frequency of subclinical hypoglycemia is lower (6–31%) in studies utilizing in-clinic BG curves with non-intensive insulin protocols, the frequency of clinical hypoglycemia is similar or higher (2% and 7%).[6,7] Clinical signs, when present, appear to be mostly neurologic in nature.[8]

In surveys that investigated the quality of life of diabetic pet owners, as well as perceived quality of life of their diabetic pets, owners' fears of hypoglycemia had one of the largest negative impacts on their quality of life.[9,10] The American Diabetes Association (ADA) recommends that glucagon is routinely prescribed to people who are at risk for severe hypoglycemic episodes.[9] Glucagon administration is recommended for use during severe hypoglycemic events, in which the patient is unable to consume glucose or other carbohydrates themselves or does not have access to such products.[11] Glucagon is not routinely prescribed to diabetic dogs and cats receiving insulin therapy, and its use in veterinary medicine is limited to the treatment of hypoglycemia refractory to intravenous (IV) dextrose administration.[12] It has already been shown that glucagon increases BG in cats; however, all available injectable glucagon formulation that are intended for emergency use (ie, ready-to-inject pens) deliver doses that consistently cause nausea and vomiting in cats.[13] As such, currently available injectable glucagon can be extremely dangerous to use in the hypoglycemic cat that is comatose and seizing.

Baqsimi (Amphastar Pharmaceuticals, Inc) is an intranasal glucagon powder medication recently approved for use in diabetic individuals for severe hypoglycemic events. Baqsimi delivers 3 mg of glucagon powder in a simple one-step dispensing device. The powder is formulated to be passively absorbed through the nasal mucosa in humans.[14] Pharmacokinetic studies of the drug in both pediatric and adult populations showed dose-responsive increases in plasma glucagon levels, with overall lower plasma glucagon levels compared with smaller intramuscular doses of glucagon without significant pharmacodynamic differences.[15,16] Acutely, glucagon administration in people decreases serum potassium concentrations.[14] Toxicology studies have been carried out using rat, dog and rabbit models, and have shown no long-term adverse effects of sub-chronic administration of the intranasal glucagon.[17] Baqsimi has not been previously studied in cats. As a transmucosal glucagon formulation, Baqsimi has the potential to be used emergently at home by owners to treat life-threatening hypoglycemia and with no need for technical expertise. Baqsimi might also improve diabetic pet owners' quality of life by reducing their fear of hypoglycemia and stress related to the potential hypoglycemia-induced death of their pets. Glucagon could be another tool for diabetic pet owners to have on hand, especially owners who do not have fast access to emergency medical care. While other glucagon formulations exist, the pricing of Baqsimi is comparable with other pre-mixed syringes of glucagon and takes less expertise to administer given that injectable glucagon is intended to be delivered intramuscularly.[18] Baqsimi has a shelf-life of 2 years.[14] While dextrose solutions or corn syrup have been historically recommended for pet owners to utilize in these situations, in an obtunded or seizing patient, oral/buccal administration of liquids pose a risk for aspiration events. Transmucosal glucagon also holds a potential benefit for in-hospital use when vascular access is not easily obtained and a severe hypoglycemic event occurs. The primary aim of this study was to evaluate if Baqsimi, administered intranasally and rectally, is effective in

raising BG concentrations in healthy cats. A secondary aim was to describe acute adverse reactions to Baqsimi administration.

MATERIALS AND METHODS

Animals

Six neutered, domestic shorthair, purpose-bred cats (four female, two male), aged 7–8 years, were included in this study. The cats were sourced from a professional vendor 3 years before the study and had been living in the cat colony at the University of Florida. All cats were overweight or obese with a body condition score range of 6–8 on a 9-point scale. The median bodyweight was 5.0 kg (range 3.9–6.2). Cats were group-housed in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International with a fixed 13–11-h light/dark cycle, ambient temperature of 22.2°C and 50% humidity. All cats were socialized and acclimatized to catheter bandages and routine handling and restraint for at least 12 months before the start of the study. Extensive environmental enrichment was provided, including 1–3h of daily human interaction and 24-h access to various toys and climbing apparatus. Cats were fed commercial dry cat food (2060 Teklad Global Cat Diet; Envigo) ad libitum in sufficient amounts to maintain body weight. Water was available for cats at all times. Cats were deemed healthy based on routine weekly physical examinations, systemic bloodwork (complete blood count and serum biochemistry panels performed approximately 2 years before the start of the study) and the absence of clinical signs of disease. Experiments were performed in ambient temperatures between 20°C and 24°C in their routine environment. All animal use was approved by the University of Florida Institutional Animal Care and Use Committee (protocol number 202300000345).

Blood glucose, glucagon and potassium measurements

All blood samples were drawn from vascular access ports (VAPs; CompanionPort CP-202K; Norfolk Vet Products) that were previously implanted for another study. The VAPs were surgically placed under general anesthesia into the jugular vein of each cat at least 3 months before beginning the experiment. VAP patency was maintained by weekly heparinized saline (10 U/ml) flushing followed by a 0.5 ml (100 U/ml) heparin lock injection, which was aspirated and discarded before sample collection. Blood glucose concentrations were measured using a handheld glucometer validated for use in cats (AlphaTrak 2 Blood Glucose Monitoring System; Zoetis). Blood samples for glucagon measurement were collected in chilled EDTA tubes, placed on ice until the end of each experiment. The samples were then centrifuged, and the plasma was separated and frozen in −80°C until analysis. Plasma glucagon concentrations (pGlucagon) were measured with a glucagon ELISA (Glucagon ELISA; Mercodia AB) validated for use in cats[19] and plasma potassium concentration (K+) was measured with a point-of-care i-STAT Alinity v (Zoetis) with i-STAT CG8+ cartridges.

Drug and placebo

Cats were passively restrained by one handler and Baqsimi (3 mg) was either administered into one nostril or rectally. For nasal and placebo application, the applicator tip was placed against the nostril so that the nostril was completely covered by the applicator. During rectal application, the tip of the applicator was inserted 5–10 mm rectally. The applicator was held between finger and thumb and the plunger was pressed as instructed by the manufacturer. After administration, empty applicators were cleaned and subsequently used for the mock application (placebo group). During placebo administration, the applicator was held against the nostril and then the back of the applicator was tapped to mimic the impact and sound created during the Baqsimi application. The Baqsimi applicator is not reusable, thus an inert powder was not able to be placed in the system to simulate a more accurate placebo experience.

Study design

This was a randomized controlled crossover study. Each cat was randomly assigned to receive one of three treatments: Baqsimi intranasally (nasal group); Baqsimi rectally (rectal group); and mock intranasal (placebo group). There was a 1-week washout period between treatments (see table in the supplementary material for the order in which each cat received the treatments). All cats were fasted overnight (>12h) before data collection. For the treatment groups, BG concentrations were measured at −15 and −1 mins before administration and 5, 15, 25, 35, 45 and 60mins after. pGlucagon was measured at −15, −1, 5 and 15mins in 5/6 cats. pGlucagon was not measured in the sixth cat as it did not complete all arms of the study (see below). K+ was measured at −1, 5, 15, 25 and 60 mins. A spectrum of fear, anxiety and stress (FAS) score[20] was measured at all time points including at the time of administration (0 min). A FAS Spectrum score of 1 indicates mild/subtle signs of fear/anxiety/stress, scores of 2 and 3 indicate moderate signs, a score of 4 indicates severe signs, where the cat may actively try to escape or may freeze, and a score of 5 indicates severe signs, where a cat may exhibit confrontational or repelling behaviors.[14] Cats were observed for 1 h after administration of the glucagon for adverse effects (sneezing, nausea, vomiting). For the placebo group, BG concentrations were measured at −15, −1, 5, 15 and 25 mins. K+ was measured at −1, 5, 15 and 25 mins. pGlucagon was not measured in the placebo group.

Statistical analysis

All data were presented as median and ranges and analyzed with non-parametric tests using commercially available computer software (GraphPad Prism; GraphPad Software, Inc). Non-parametric repeated measure analyses were used for all comparisons. Results from time points T−15 and T−1 were averaged and presented as T−8. T−8, T15 and T25 were compared within each treatment group using Friedman tests with adjusted P values (Dunn's multiple comparison tests) reported. Statistical significance was defined as $P < 0.05$.

RESULTS

Drug application and tolerability

A total of six cats were enrolled in this study. All cats completed the entire study except for one that did not receive the rectal administration after its first two procedures owing to reasons unrelated to the study protocol (see supplementary figures 1–3 showing individual cat data for each treatment in the supplementary material). Two of six cats (cats 5 and 6) in the nasal treatment group had unsuccessful medication applications, during which most of the powder aerosolized. In cat 5, the powder was pushed intranasally but 1 s later was forcibly exhaled. In this cat, there was only a marginal change in BG concentration. In cat 6, the cat turned its head during intranasal deployment of the Baqsimi applicator and the authors were unsure if any medication successfully entered the cat's nose. This failed application was associated with no change in BG concentrations. There was a trend toward increased FAS scores from baseline (median 0, range 0–1) to time of application of nasal (median 4, range 2–5; $P = 0.06$) and placebo (median 3, range 2–4; $P = 0.06$), but not during rectal administration (median 0, range 0–2; $P = 0.5$). Five minutes after administration, all FAS scores were in the range of 0–2 for all groups. No additional restraint aids aside from the person passively restraining the cat were required in any treatment groups.

Adverse reactions are reported in Table 1. Sneezing was the most common reaction for cats receiving intranasal administration (4/6 cats), followed by hypersalivation (3/6 cats) and blepharospasm (2/6 cats). Sneezing occurred within seconds of nasal administration of the medication and resolved within 5mins in all cats. One of the cats that sneezed was cat 6 that turned its head during drug administration. After rectal administration, 2/5 cats vomited; one cat vomited 11 mins after administration and the other vomited 60 mins after administration. There was one episode of vomiting in the placebo group 34 mins after administration.

Table 1 Summary of adverse reactions by study group

Glucagon or placebo	Route of administration	Total number of cats	Vomiting	Hypersalivating	Sneezing	Blepharospasm
Glucagon	Intranasal	6	0	3	4	2
Glucagon	Rectal	5	2	0	0	0
Placebo	Intranasal	6	1	0	0	0

Data are n

Drug efficacy

BG concentrations were available for cats at all time points. K+ was available for all scheduled time points, except for T−1 in two cats in the nasal treatment group, due to malfunction of the iStat. Baseline BG, K+ and pGlucagon did not differ between treatment groups. Overall median baseline BG concentrations were 101 mg/dl (range 91–110). Overall median baseline K+ was 3.6 mmol/l (range 3.5–4.1). Overall mean baseline pGlucagon concentration was 8.5 pmol/l (range 2.9–41.2). pGlucagon was only tested in the treatment groups due to limited study funds (Figure 1). Cat 6 (the cat that did not complete the rectal portion of the study and turned its head during the nasal portion of the study) did not have glucagon measured after nasal administration. In the nasal group (n = 5), there was an increase in median pGlucagon from 12.2 mmol/l (range 3.5–44.1) at baseline (T−8) to 260.1 mmol/l (range 7.2–349.8) at T5 (P=0.05) and to 218.5mmol/l (range 7.9–349.8) at T15 (P=0.02). In the rectal group (n=5), there was an increase from 6.9 mmol/l (range 2.9–21.1) at baseline (T−8) to 349.8mmol/l (range 30.5–349.8) at T5 (P=0.08) and to 349.8 (range 67.4–349.8) at T15 (P = 0.01). For each treatment group, BG concentrations increased in both the nasal and rectal treatment groups as early as 5 mins after administration but did not change after placebo (Figure 2). After nasal Baqsimi administration (n=6), BG concentrations increased at 15 mins (P=0.0064) to 137.5mg/dl (range 104–251). After rectal Baqsimi administration (n = 5), BG concentrations increased at 15 mins (229 mg/dl, range 99–285; P = 0.002) and 25 mins (223 mg/dl, range 94–291; P=0.002). There was no increase in BG in 3/6 cats after nasal administration (two of which were observed to not receive the full dose, as explained above) and in 1/5 cats after rectal administration. In the cats that did respond to the nasal administration of Baqsimi, BG increased by ≥20mg/dl within 5mins. In the four cats that responded to rectal administration, BG concentration increased by ≥20 mg/dl within 15 mins; in 3/4 cats, this was observed in 5 mins. In the placebo group, there was no change in K+ (Figure 3). In the nasal group (n=4; cats 1 and 6 were excluded from the statistical analyses owing to an error in baseline K+ measurements), there was a mild decrease in K+ at T15 from 3.8mmol/l (range 3.6–4.1) to 3.4mmol/l (range 3.1–3.6; P=0.02) (Figure 3). In the rectal group (n = 5), there was a mild decrease in K+ from 3.7 mmol/l (range 3.5–3.9) to 3.2 mmol/l (range 3.1–3.6) at T15 (P = 0.04) and to 3.1 mmol/l (range 2.9–3.4) at T25 (P = 0.01) (Figure 3).

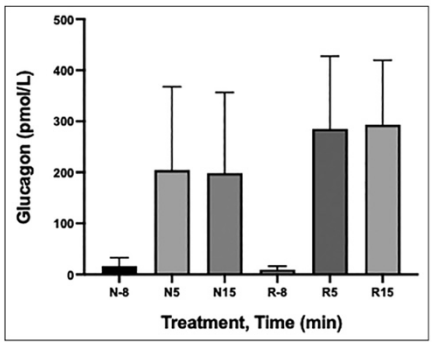

Figure 1 Bar graph showing medians and ranges for plasma glucagon concentrations in the nasal (N) and rectal (R) treatment groups at time points T−8 (average of T−15 and T−1), T5 and T15 mins

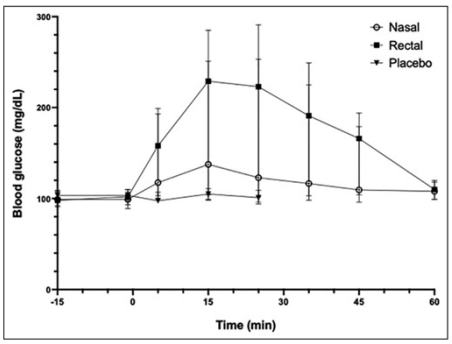

Figure 2 Line graph of median and ranges of blood glucose concentrations for nasal group (open circle), rectal group (black square) and placebo (black triangle)

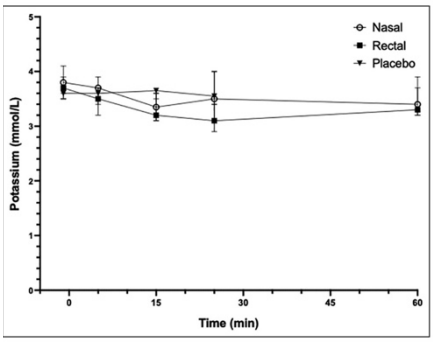

Figure 3 Line graph of median and ranges of plasma potassium concentrations for nasal group (open circle), rectal group (black square) and placebo (black triangle)

DISCUSSION

This study shows that Baqsimi, when administered intranasally and rectally, can increase BG concentrations within minutes and is tolerated in healthy cats with only mild and very transient adverse effects. FAS scores were similar between the placebo group and the nasal administration group, and lower in the rectal administration group, demonstrating greater tolerability of the rectally administered medication. The inclusion of an intranasal control group with demonstratable increases in stress without corresponding changes in BG was utilized to show that BG changes after drug administration (nasal and rectal) were the result of the medication itself rather than stress. Rectal administration of Baqsimi was associated with greater increases in BG and glucagon concen- trations; however, it is unclear to what extent this was related to failed drug administration in the nasal group vs decreased absorption. In both groups, the rapid response to Baqsimi was similar to that previously reported for IV and intramuscular glucagon administration, with a peak in BG concentrations after 15 mins.[13]

The frequency of vomiting seen with Baqsimi administration was lower than in studies describing IV glucagon administration in cats.[13] In human trials of Baqsimi, the most common adverse effects included nausea, headache, vomiting and upper respiratory irritation.[13–15] Vomiting is an important side effect of glucagon administration given that hypoglycemic, obtunded patients may not be able to protect their airways. Larger studies, of hypoglycemic diabetic cats, are required to further investigate the frequency of vomiting in this population and to further evaluate the risk-benefit ratio in cats suffering from life-threatening hypoglycemia.

In people, hypokalemia requiring supplementation has been noted as a possible adverse event of glucagon overdoses.[14] Although clinically relevant decreases in K+ were not observed in our study, it is important to recognize that our study population was small and relatively homogenous; therefore, potential complications of this drug might be underestimated. Larger studies, of client-owned, ideally diabetic cats, are required to further investigate the risk of hypokalemia and the risk-benefit ratio in cats suffering from life-threatening hypoglycemia. Glucagon administration has also been reported to cause a transient hyperkalemia, which was not observed in this study but might become clinically significant in a larger, more heterogenous population.21

Two of six cats in the nasal treatment group had unsuccessful medication applications, during which most of the powder aerosolized. During rectal administration, however, one of these cats showed a strong increase in BG and pGlucagon, while the other cat did not receive the rectal administration. In the authors' opinion, the lack of compliance by the cat, rather than a failure of drug absorption, may have played a role in the ineffectiveness of nasal administration in these two cases. The lack of compliance may be a minor issue in the clinical setting, as cats experiencing severe, clinical hypoglycemic episodes might be too obtunded to resist a Baqsimi application.

While the cost of Baqsimi might deter some owners, it is important to emphasize that its purchase is likely a one-time expense. The shelf-life of Baqsimi is approximately 2 years from the manufacture date, and owners should be properly educated to use it only if their cat is suffer- ing from a life-threatening insulin overdose with clinical signs such as severe obtundation, coma or seizures.

Sneezing might have decreased drug availability for absorption after nasal application. However, even if the drug was available, decreased absorption after success- ful administration might also contribute to lack of effect. In cat 3, in which BG and pGlucagon did not increase substantially after uneventful nasal administration, there was a substantial increase in both analytes after rectal administration. This was likely the result of a substantial difference in glucagon absorption through the nasal vs rectal mucosa. The difference in absorption could be related to differences in surface area or differences in the properties of the mucosa. Interestingly, in people, rectal administration of glucagon did not result in a significant change in BG concentrations, despite a significant increase in pGlucagon.[22] This might be explained by

differences in absorption into systemic and portal circulations or by stimulation of gut and pancreatic hormones that might occur with one route of administration but not the other. This could potentially be further elucidated by measuring insulin concentrations at the time of administration. Because our study was attempting to answer a clinical question on the feasibility of the use of Baqsimi, and because of limited resources available for this study, insulin concentrations were not measured. In practice, the effect of glucagon, administered by any route, on insulin secretion, is irrelevant for the following reasons: (1) by definition, insulin concentrations are expected to be excessive in the target population; and (2) within the target population, the insulin concentration is not expected to change with glucagon administration as the source of excess insulin is exogenous.

As mentioned above, one limitation of this study is the small sample size, which inherently increases the chance of a type II error. In addition, we studied purpose-bred cats that are fairly homogenous in their genetic makeup and very homogenous in their environment (including diet). Sample size and heterogeneity are especially important in assessing the array of potential side effects to a medication. The small sample size also limits the study's ability to determine whether Baqsimi treatment would routinely be useful for the treatment of hypoglycemia in cats because not all cats had a change in BG despite apparently successful medication administration. This also highlights that one dose may not be enough to restore euglycemia, if this drug restores it at all in hypoglycemic diabetic cats. The exact frequency and timing of vomiting, for example, might be an important question to investigate in future studies, as well as the exact benefit of a rapid at-home response to hypoglycemia. It is possible that the risk of glucagon-induced vomiting might outweigh the benefit of rapid resolution of hypoglycemia. Another limitation is the inability to account for how much medication was administered to each cat. This can lead each patient to receive different amounts of medication, which could lead to variation in study results.

CONCLUSIONS

This study showed that transmucosal glucagon powder administration was effective in raising BG concentrations rapidly in healthy cats with minimal side effects. Future larger studies are needed to quantify the efficacy and safety of transmucosal glucagon in diabetic cats, especially during hypoglycemic crises.

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3.5 | FreeStyle Libre 3 provides clinically accurate continuous glucose monitoring in diabetic cats

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ABSTRACT

Objectives

FreeStyle Libre 3 (FSL3) continuous glucose monitoring system (CGMS) is accurate in diabetic people and its smaller size could be advantageous for use in veterinary patients. The aim of this study was to assess the analytical and clinical accuracy, sensor lifespan, and incidence of complications associated with the FSL3 in diabetic cats.

Methods

In this prospective study, interstitial glucose concentrations (IG) measured by FSL3 were compared to blood glucose concentrations (BG) measured by AlphaTRAK2. Skin reactions at the application site and sensor lifespan were recorded. Pearson's correlation coefficient, Bland-Altman test, and Parkes Error Grid analysis (EGA) were used to evaluate correlation, bias, and clinical accuracy, respectively. Analytical accuracy was assessed using the mean absolute relative difference (MARD).

Results

Median sensor lifespan was 9 days (range, 4–14). After sensor removal, 2 cats had a mild erythema at the application site. A total of 210 paired BG-IG measurements were available for analysis, the majority (203/210, 96.7%) falling within the euglycemic (70–180 mg/dL) and hyperglycemic (>180 mg/dL) ranges. A strong positive correlation was observed between IG and BG readings ($r = 0.95$; $P < 0.0001$). Comparison of IG and BG measurements resulted in a MARD of 13.4% and an overall bias of -25.4 ± 45.4 mg/dL. Clinical accuracy was demonstrated, with 99.5% (209/210) of the results in zones A+B of the Parkes EGA.

Conclusions and Relevance

Freestyle Libre 3 provides clinically accurate measurements in the euglycemic and hyperglycemic ranges in diabetic cats. The shortest sensor lifespan was longer than that reported in earlier studies using previous FSL models. The smaller size of the FSL3 might offer advantages in diabetic cats, potentially improving adherence and long-term use of CGMS.

INTRODUCTION

Insulin treatment necessitates close monitoring in cats with diabetes mellitus (DM).[1,2] When dysglycemia is effectively managed, glucotoxicity is minimized and cats are more likely to achieve diabetic remission.[3-5] In the last decade, continuous glucose monitoring systems (CGMS) have revolutionized the management of DM in both human and veterinary medicine.[6,7] These devices measure interstitial glucose concentrations (IG) on a minute-by-minute basis over consecutive days or weeks, reducing blood sampling-associated patient discomfort and greatly increasing information on glucose fluctuations and trends.[6-8] The FreeStyle Libre (Abbott Laboratories) is currently the most studied CGMS in veterinary patients. The accuracy of the first (FSL1) and the second generation (FSL2) has been previously evaluated in healthy and diabetic cats.[9-13] Despite the good clinical accuracy, the premature detachment of the sensor represents one of the most frequent complications in diabetic cats,[14,15] with a median time of sensor activity ranging from 5 to 10 days.[9-12] In 2020, a third generation of the device, FreeStyle Libre 3 (FSL3), has been licensed for use in diabetic people. The FSL3 measures IG using the same sensing technology as the FSL2. Similar to the FSL2, the FSL3 provides continuous IG readings every minute, along with real-time glucose levels, trends, and alerts.[16] However, the FSL3 features a newly designed one-piece applicator, lasts longer (up to 15 days) and the sensor is approximately 60% smaller than previous FSL models.[16] A recent study demonstrated that the FSL3 provides accurate glucose measurements across a broad glycemic range in diabetic people.[16] The smaller size of the FSL3 could provide significant advantages for veterinary patients, particularly in cats. However, no studies have evaluated the performance of the FSL3 in veterinary diabetic patients. The aim of this study was to assess the analytical and clinical accuracy, sensor lifespan, and incidence of complications associated with the FSL3 in diabetic cats.

MATERIALS AND METHODS

Animals

Fourteen client-owned diabetic cats were enrolled. Diagnosis of DM was in accordance with the Agreeing Language In Veterinary Endocrinology criteria established by the European Society of Veterinary Endocrinology.[17] Five neutered females and 9 neutered males domestic shorthair cats were included. Median (range) age was 11.5 (7-16 years). Median body weight was 4.1 kg (2.6-7.2 kg) and median (range) body condition score was 4.5/9 (3/9-6/9). Seven cats were treated with insulin glargine 100 U/mL and 6 with insulin glargine 300 U/mL. In one case, insulin therapy was discontinued as the cat was considered to have achieved diabetic remission. The median insulin dose was 4 (range, 0-10) U/cat/day. Two cats had concurrent hypersomatotropism and one of them was receiving cabergoline; one cat had inflammatory bowel disease, and another cat had chronic kidney disease along with feline immunodeficiency virus infection and was fed with a renal prescription diet.

Protocol and informed consent forms were approved by the Scientific Ethics Committee of the University of Bologna (protocol number 296281). Recruitment of cats to the study was voluntary and at no cost to the owners. Written informed consent was obtained before enrollment in the study.

Accuracy of FSL3

Accuracy of the FSL3 was assessed by comparison to a veterinary portable blood glucose meter (vPBGM; AlphaTrak2, Blood Glucose Monitoring System, Zoetis srl, Rome, Italy) previously validated for use in cats with a BG range 20-750 mg/dL and intra-assay coefficient of variation of 3.8%.[18] In order to compare IG measured with FSL3 to the BG obtained with vPBGM, paired samples were collected and then classified as being in the hypoglycemic (<70 mg/dL), euglycemic (70-180 mg/dL), or hyperglycemic range (>180 mg/dL). All concentration above and below the detection limit of the sensor (≤20 and ≥500 mg/dL) were excluded from the statistical analysis. During the wearing period of the sensor, each cat was evaluated for 3 time periods in hospital, each lasting 12 hours, as follows: 1st day, 5-7th, and

12nd-14th day. On day 1 of the study, cats were hospitalized after food and insulin were administered at home. The FSL3 sensor (Abbott Laboratories Ltd, Chicago, Illinois) was placed as previously described[11] immediately after arrival in the hospital. The sensor was placed on the dorsal or lateral aspect of the neck or, if application in the neck area was not possible, the sensor was placed caudally on the dorsum. A drop of glue (Loctite Super Attak, Henkel Italia Srl, Milan, Italy) was applied to the skin-facing surface of the sensor in all diabetic cats. When positioned on the neck, the sensor was secured with a cotton and elastic bandage (Figure 1). Glucose measurements were started 1 hour after the sensor was applied (period of initialization). During the second (day 5-7th) and third evaluation (day 12nd-14th) periods, cats were hospitalized after food and insulin were administered at home and glucose measurements were started immediately after arrival in the hospital. Interstitial glucose concentration was recorded using a smartphone connected to the FSL3 at the same time as each BG was measured by the vPBGM, and both were recorded as paired values. At the end of the wearing period, sensors were removed by a single clinician in the hospital or by owners at home. If the sensor was removed at home, owners were asked to photograph the skin in the area where the sensor was applied. The skin in that area was evaluated (either directly or by viewing the photographs) subjectively by a single clinician for the presence of erythema or any other dermatological abnormalities. Sensor lifespan was defined as the duration from sensor application to the cessation of IG reading activity due to any cause (e.g., removal of the sensor by the cat, sensor malfunction).

Figure 1: FreeStyle Libre 3 (FSL3) application in a diabetic cat. (1) the dorsal aspect of the neck is trichotomized; (2) the skin is cleaned with chlorhexidine wipes; (3) the FSL3 has a one-piece sensor applicator, the cap is unscrewed from applicator; (4) the cap is set aside, the sensor applicator is ready; (5) a drop of glue is added on the skin-surface of the sensor; (6) the sensor applicator is placed over the site and pushed down firmly (listening for the 'Click') in order to apply the sensor; (7) it is ensured that the sensor is secure (if necessary, the forceps can be used to avoid the detachment of the sensor); (8) the sensor is attached to the skin, the applicator is pulled back slowly; (9) the sensor is additionally secured by covering it with a patch; (10) The Freestyle Libre 3 app is opened on the smartphone, selecting 'Scan New Sensor' and holding the top of the smartphone near the sensor to activate it; (11) the sensor is secured with a cotton and an elastic bandages, and (12) the cat is ready to go home.

Data Analysis

Statistical analysis was performed using a commercially available computer software program (GraphPad Prism version 10.1.1). Normality was assessed using the Shapiro-Wilk test, and nonparametric tests were used accordingly. The Wilcoxon test was used to compare IG and BG measurements. The correlation between BG and IG was assessed using Pearson's r test. Proportional bias between BG and IG measurements was assessed using the Bland-Altman test. Accuracy was evaluated according to ISO 15197:2013 guidelines, with acceptable analytical accuracy defined as 95% of IG results being within 15 mg/dL (when BG ≤100 mg/dL) or 15% (when BG >100 mg/dL) of paired BG, and clinical accuracy as ≥99% of IG falling in zones A or B of the Parkes Error Grid analysis (EGA) as formulated for people with Type I DM.[19]

Analytical accuracy was determined by calculating the mean absolute relative difference (MARD), median absolute relative difference (mARD), and mean absolute difference (MAD).[20] Statistical significance was set at $P < 0.05$.

RESULTS

Application of the sensor was successful and easy to perform in all cats. In 13/14 cats, the sensor was placed on the dorsal or lateral aspect of the neck, while, in one cat, it was placed more caudally on the dorsum, as it was better tolerated. The median (range) sensor lifespan was 9 (4-14) days. Sensor lifespan was 14 days in 3 cats, 11 days in 2 cats, 10 days in one cat, 9 days in 3 cats and 8, 7, 6, 5, and 4 days in one cat each. The FreeStyle Libre was applied for the first time in 7/14 (50%) cats included in the study, while the remaining 7/14 (50%) cats had prior experience with the sensor. Among the latter group, one cat had used the sensor once, another four times, three cats five times, one cat nine times, and one cat more than ten times. The three cats with sensor lifespan of 14 days had previously used the device (five times in one cat, nine times in another, and more than ten times in the third). In 11/14 cats it was not possible to perform the third evaluation of the study due to sensor removal by the cat (8/11) or sensor malfunction (3/11). After sensor removal, 2 cats had a mild erythema at the application site.

A total of 210 paired BG and IG data points were recorded, of which 64.3% (135/210) were in the hyperglycemic range as determined by the vPBGM (BG, 372 mg/dL; range, 182-585); 32.4% (68/210) were in the euglycemic range (BG, 125 mg/dL; range, 71-179) and 3.3% (7/210) were in the hypoglycemic range (BG, 59 mg/dL; range, 46-68). Median (range) glucose concentrations in all measured samples were 240.5 (53-499) mg/dL using the FSL3, and 269.5 (46-585) mg/dL using the vPBGM ($P < 0.001$). Interstitial glucose readings underestimated 159/210 (76%) and overestimated 49/210 (23%) BG readings measured by the vPBGM, while identical values were recorded in 2 IG-BG paired readings.

A strong positive correlation was found between IG and BG readings ($r = 0.95$; 95% confidence interval [CI], 0.93-0.96 $P < 0.0001$; Figure 2). Data evaluation using the Parkes consensus EGA showed that 99.5% (209/210) of the samples were in the zones A and B (Figure 3).

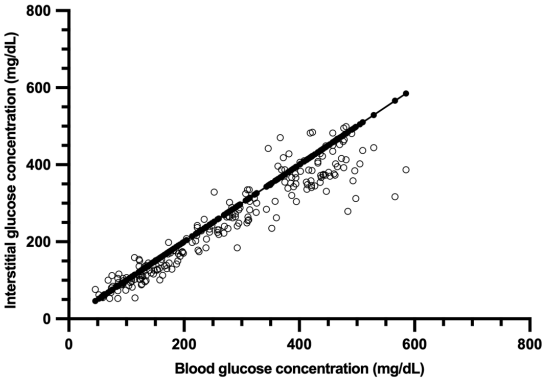

Figure 2: Correlation between blood glucose and interstitial glucose concentrations in diabetic cats (n = 14). Blood glucose measured by AlphaTrak2. Interstitial glucose measured by FreeStyle Libre 3.

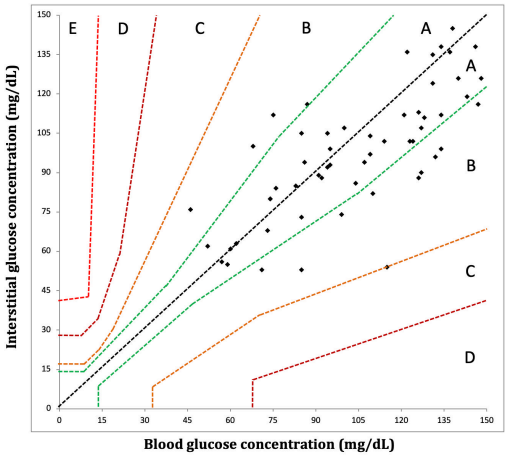

Figure 3: Parkes Error Grid analysis exhibiting excellent clinical accuracy of FSL3 in diabetic cats (n = 14). The 99.5% percent of data points were within zone A (indicating no change in clinical action) or zone B (indicating change in clinical action unlikely to affect outcome), with 93.3% (n = 196) in A and 6.2% (n = 13) in B. Blood glucose measured by AlphaTrak2. Interstitial glucose measured by FreeStyle Libre 3.

The mean ± standard deviation (SD) differences between the IG and the BG were -25.4 ± 45.4 mg/dL (95% limits of agreement, −114.4–63.5; Figure 4). The results of FSL3 analytical accuracy in the BG ranges at or below 100 mg/dL and above 100 mg/dL are presented in Table 1.

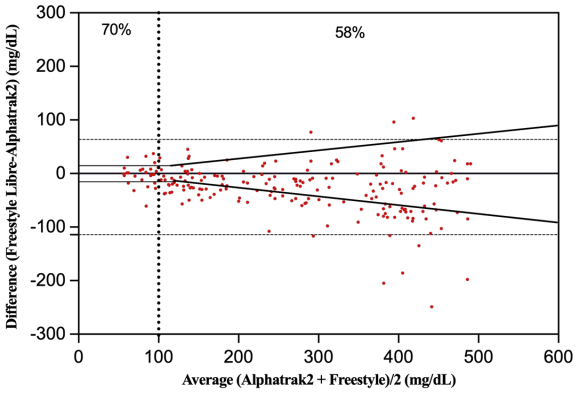

Figure 4: Bland Altman plot of agreement between blood glucose and interstitial glucose concentrations in diabetic cats (n = 14). The standard required limits are defined by the black symmetric line: at ±15 mg/dL from the reference value for glucose determinations ≤ 100 mg/dL and ±15% from the reference value for glucose determination >100 mg/dL. Percentages express the number of samples within the limits when the reference determination was ≤ or >100 mg/dL. Blood glucose measured by AlphaTrak2. Interstitial glucose measured by FreeStyle Libre 3.

Blood glucose concentrations ≤ 100 mg/dL	
Number of glucose values	27
MAD (mg/dL)	23.1
Percent of values within ±15 mg/dL of the BG value	70% (19/27)
Blood glucose concentrations > 100 mg/dL	
Number of glucose values	183
MARD (%)	13.4
mARD (%)	13.5
Percent of values within ±15% of the BG value	58% (106/183)

Abbreviations: MAD, mean absolute difference; MARD, mean absolute relative difference; mARD, median absolute relative difference.

Table 1. Results of Freestyle Libre 3 analytical accuracy for blood glucose concentrations ≤ 100 mg/dL and > 100 mg/dL.

DISCUSSION

Our study is the first to evaluate the performance of the FSL3 in cats with DM. Although it does not fulfill the analytical ISO 2013 accuracy requirements, the FSL3 demonstrated acceptable clinical accuracy to be used as an IG monitoring tool in diabetic cats. The use of CGMS represents a paradigm shift in veterinary diabetic management. These devices allows real-time, comprehensive assessment of glucose fluctuations throughout the day and night, as well as glucose trends over multiple days, enabling clinicians to make faster and more informed decisions regarding insulin dose adjustments.[6,21] However, monitoring with CGMS may be more costly than simpler monitoring methods, particularly if the sensor is not well tolerated by the cat. Premature sensor detachment is a commonly reported complication in diabetic cats, with a median sensor lifespan ranging from 5 to 10 days.[9-12] Moreover, in a recent survey, one of the primary concerns expressed by owners of diabetic pets, especially diabetic cats, was the shortened lifespan of the FSL sensor.[15] The most recent version of the FreeStyle Libre 3 (Freestyle Libre 3 Plus, Abbott Laboratories) is designed for a 15-day wear period. In this study, we evaluated the FSL3 before this update, limiting the availability of IG readings to a maximum of 14 days. In our cohort of diabetic cats, the median (range) time of sensor activity was 9 (4–14) days. These findings demonstrate an improvement compared to our previous study using the FSL1, where the median sensor lifespan was notably shorter, at 5.5 (1–14) days.[11] However, the median sensor lifespan reported in this study is consistent with other reports evaluating previous generations of the FreeStyle Libre in diabetic cats, which reported a median duration of 7 to 10 days.[9,10,12] Notably, the shortest sensor lifespan in our study (4 days) was longer than that reported in earlier studies (1–2 days).[9-12] The reasons for this discrepancy are speculative; however, our findings may suggest improved tolerability of the FSL3, potentially due to its smaller size. However, it is possible that the cats in our study were more accustomed to wearing the sensor from prior applications, which may have reduced the likelihood of premature detachment. Also, direct comparison with other studies is limited by differences in sensor placement (neck vs dorsum or thoracic wall), application methods (glue and bandage vs only glue or skin sutures), and variation in sampling environments and

frequencies (home-based vs hospital-based settings).[9-12] In the authors' opinion, while it is desirable for the FSL to remain in place for the full duration of sensor activity, even 1-3 days of continuous IG readings offer a more comprehensive assessment of diabetes management compared to a traditional 12-hour BGC or the measurement of serum fructosamine concentration. Therefore, despite potential issues with sensor tolerance in some cats, the FSL remains the preferred method for monitoring diabetic patients in the authors' clinical practice. Dermatologic complications associated with the use of FSL have been reported in up to 18% of diabetic cats, ranging from mild (erythema, crusting, abrasions, mild pruritus, discomfort) to severe (erosions, ulceration, abscess formation, severe pruritus).[14] In our study, only 2 cats showed mild dermatologic changes (erythema at the sensor application site), despite the use of additional glue. Importantly, these dermatologic changes were clinically inconsequential and did not interfere with the placement of subsequent FSL sensors.

We found a good clinical accuracy of the FSL3, with a strong positive correlation between IG and BG, consistent with previous studies evaluating the performance of FSL1 and FSL2 in diabetic cats.[9-11] The accuracy of the FSL3 is reported here based on 210 paired BG-IG measurements, the majority of which (96.7%) were in the euglycemic (70-180 mg/dL) and hyperglycemic (>180 mg/dL) ranges. Due to the limited number of IG readings below 70 mg/dL (3.3%), no definitive conclusions can be drawn regarding the accuracy of the FSL3 in the hypoglycemic range. Further studies are warranted to evaluate the device's performance in hypoglycemic cats.

Similar to previous veterinary studies utilizing the ISO 2013 guidelines, our study did not meet the standards for analytical accuracy. However, applying ISO criteria while comparing two distinct compartments (blood and interstitium) might be inappropriate because of the physiological differences between these compartments. Consequently, the observed discrepancy between values obtained from both methods might not solely reflect an actual inaccuracy of the FSL. Part of this discrepancy might be attributed to the estimated time lag (5-11 minutes) required for equilibration between the blood and interstitial fluid.[11,22] Additionally, stress-induced hyperglycemia is a well-recognized phenomenon in cats, and fluctuations in BG concentrations can occur within minutes of stress induction.[23,24] Therefore, it is plausible that the act of removing the cat from its cage to measure BG induces stress, leading to an increase in capillary glucose concentration, while the IG concentration remains in the equilibrium phase. This could explain why the FSL3 underestimated BG values in 76% of the cases. We therefore hypothesize that in the cat’s natural environment, the FSL3 would actually perform with greater accuracy, although this would be difficult to confirm.

In human diabetology, to address the limitations of ISO standards, MARD has become the most widely used metric for evaluating the analytical accuracy of CGMS.[25,26] The MARD is calculated by averaging the absolute values of the relative differences between CGMS measurements and the corresponding reference method results. In this context, "absolute" indicates that each relative difference is considered as a positive value, regardless of whether the difference compared to the reference result is positive or negative.[25] A lower MARD percentage indicates that CGM readings are closely aligned with the reference glucose values, whereas a higher MARD percentage reflects greater discrepancies between the CGMS and the reference method.[26] The MAD is a similar metric, but it reports the magnitude of the difference in absolute terms, rather than as a percentage, and is often used to assess accuracy at lower BG levels (≤ 100 mg/dL). Although controversy exists regarding the exact cut point for accuracy, MARD values below 14% are generally considered acceptable in human studies.[27] In our study, when IG readings were compared to BG measurements obtained via the vPBGM, the overall MARD was 13.4%. This value is higher than the overall MARD reported for the FSL3 in diabetic humans (7.8%).[16] However, a direct comparison between the two studies is not feasible because of the differences in the number of IG-BG paired readings (210 vs. 6845), reference methods (vPBGM vs. glucose analyzer), glucose ranges (with most values <350 mg/dL in the human study), and species-specific factors, such as the higher susceptibility of cats to stress-induced hyperglycemia. Few studies have assessed MARD in veterinary diabetic patients.[28-30] Malerba and

colleagues evaluated the FSL1 in dogs with diabetic ketoacidosis (DKA), reporting MARD values of 19.7% before and 17.2% after DKA resolution.[28] In a recent study evaluating the FSL in dogs under one year of age with systemic illness, the overall MARD was 15.4%.[30] Additionally, in a previous canine study using a 180-day implantable glucose monitoring system, the overall MARD was 24.5%.[29] In the present study, the MARD value is lower than those reported in the aforementioned veterinary studies. However, differences in MARD values across studies might be attributed to variability in study design, patient selection, comparison methods, and other factors.[31] Furthermore, the paucity of studies in veterinary settings complicates establishing an appropriate cut-off value for the MARD that would indicate acceptable accuracy in diabetic veterinary patients. Considering the need for much tighter glycemic control in people compared to veterinary patients, it is unlikely that MARD cutoffs would need to be as low in veterinary diabetic patients. Therefore, it is recommended that future studies evaluating CGMS accuracy in diabetic veterinary patients include MARD assessments to facilitate comparisons across a broader range of reports.

Limitations of this study include the small number of paired samples, as the third evaluation could not be performed in a significant number of cats. Another important limitation is the low number of data points in the hypoglycemic range, which is crucial for clinical decision-making. Thus, further studies are required to assess accuracy in this range. Additionally, the study was unable to evaluate precision or compare the optimal sensor location, as only one sensor was applied in each cat. Lastly, accuracy over time was not assessed.

CONCLUSIONS

Freestyle Libre 3 provides clinically accurate measurements in the euglycemic and hyperglycemic ranges in diabetic cats. The device was well tolerated by diabetic cats, and the shortest sensor lifespan was longer than that reported in earlier studies using previous FSL models. The smaller size of the FSL3 might offer advantages in diabetic cats, potentially improving adherence and long-term use of CGMS. Although the FSL3 may not remain on the cat for the entire duration of sensor activity, in most cases, IG data can be obtained over several days and nights, allowing for a thorough assessment of diabetes management. Additional studies are warranted to determine whether long-term use of the FSL3 improves glycemic control and outcome compared to traditional monitoring methods, such as BGCs, in diabetic cats.

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3.6 | Accuracy of the FreeStyle Libre 3 continuous glucose monitoring system in hypo- and euglycemic cats

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ABSTRACT

Background

The FreeStyle Libre 3 (FSL3) has several improvements compared to previous FreeStyle Libre systems, but its accuracy has not yet been determined in cats. In diabetic people, FSL3 offers increased accuracy, and its smaller size could be advantageous for use in veterinary patients.

Objectives

Assess the accuracy of FSL3 in cats with experimentally-induced hypoglycemia.

Animals

Seven healthy, purpose-bred cats.

Methods

Hyperinsulinemic-hypoglycemic clamps were performed. Interstitial glucose concentration (IG), measured by FSL3, was compared to blood glucose (BG) measured by AlphaTrak2. Data were analyzed for all paired measurements (n = 474) and during stable BG (≤1 mg/dL/min change over 10 minutes). Pearson's r test, Bland-Altman test, and Parkes Error Grid analysis (EGA) respectively were used to determine correlation, bias, and clinical accuracy.

Results

Blood glucose and IG correlated strongly (r = 0.86, P<.0001) in stable glycemia and moderately at all rates of change (r=0.73, P<.0001). Analytical accuracy was not achieved, whereas clinical accuracy was demonstrated with 99-100% of the results in zones A+B of the Parkes EGA. Interstitial glucose concentration underestimated BG in euglycemia and mild hypoglycemia (mean -11.7±11.2, -5.5±9.1, -1.5±6.0 mg/dL in the ranges 91-120, 66-90, and 56-65 mg/dL, respectively), but overestimated BG in marked hypoglycemia (mean 6.3±5.7, 15.7±5.6 mg/dL in the ranges 46-55 and <45 mg/dL, respectively).

Conclusions

The FSL3 underestimate BG across most of the hypo-euglycemic range but overestimates BG in marked hypoglycemia (<55 mg/dL). Recognizing the proportional, glycemic-dependent bias of FSL3 improves the safety of its clinical application in feline patients.

INTRODUCTION

Continuous glucose monitoring systems (CGMS) have revolutionized the management of diabetes mellitus (DM) in both human and veterinary medicine.[1,2] These devices measure interstitial glucose concentrations (IG) on a minute-by-minute basis over days or weeks, reducing blood sampling-associated patient discomfort and greatly increasing information on glucose fluctuations and trends.[2-4] Their use improves detection of hypoglycemia in both human[5-7] and veterinary patients,[3] offering a way to address primary stressors reported by owners.[8,9]

The FreeStyle Libre (Abbott Laboratories Ltd, Chicago, Illinois) is the most commonly studied CGMS in veterinary patients. The accuracy of the first generation of the FreeStyle Libre (FSL1) has been previously evaluated in both healthy and diabetic cats.[4,10-12] Interstitial glucose concentrations measured by the FSL1 correlate well with blood glucose concentration (BG) in the eu-hyperglycemic range, but interpretation in the hypoglycemic range is limited by very small sample sizes.[4,10-12] The second generation of FreeStyle Libre (FSL2) was updated with a new glucose algorithm that provided improved accuracy across the measurement range in people, specifically at the low end of the dynamic range.[13] In purpose-bred cats, FSL2 underestimates BG throughout most of the hypo-euglycemic range and generally overestimates BG in marked hypoglycemia (<50 mg/dL).[14]

In 2020, a third generation of the device, FreeStyle Libre 3 (FSL3), was licensed for use in diabetic people. The FSL3 uses the same sensing technology as the FSL2 to measure IG. Like FSL2, the FSL3 provides continuous IG readings every minute, as well as offering glucose levels, trends and alerts.[15] However, the FSL3 lasts longer (15 days), has a one-piece sensor applicator, and the sensor is about 70% smaller than FSL1 or FSL2.[15] Moreover, the FSL3 automatically sends the results to a smartphone without requiring users to scan the sensor to to obtain a glucose result. In contrast, the FSL1 and FSL2 are intermittently scanned or “flash” CGMS that requires users to scan the sensor with a smartphone or reader device. In a recent study, the FSL3 demonstrated accurate performance across the dynamic glycemic range in diabetic people.[15] In the hypoglycemic range (<70 mg/dL), this device showed good accuracy with 95% IG values within ± 20 mg/dL of the BG reference method, but the evaluation at glucose levels <54 mg/dL was limited by the small numbers of IG-BG pairs.[15] The performance and smaller size of the FSL3 could be advantageous in veterinary patients, and clinical trials are warranted. The objective of this study was to determine the analytical and clinical accuracy of the FSL3 in cats with experimentally-induced hypoglycemia.

MATERIALS AND METHODS

Animals

Seven neutered, purpose-bred, domestic shorthair cats (5 female, 2 male) were included, with median (range) ages of 7 (6-7) years. Median body weight was 4.9 kg (3.9-6.2 kg) and median body condition score was 7 (6-8) on a 9-point scale. Cats were group-housed in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All cats were socialized and acclimatized to catheter bandages and routine handling and restraint for at least 3 months before the start of the study. Extensive environmental enrichment was provided, including 1 to 3 hours of daily interaction with humans and 24-hour access to various toys and climbing apparatus. Cats were fed commercial dry cat food (Envigo 2060 Teklad Global Cat Diet) ad libitum in sufficient amount to maintain body weight. Cats were deemed healthy based on routine weekly physical examinations, annual systemic blood tests (CBC and serum biochemistry panels), and the absence of clinical signs of disease. Experiments were performed at ambient temperatures between 20 and 24 C in the cats' routine environment. All animal use was approved by University of Florida Institutional Animal Care and Use Committee (protocol number 202300000345).

Vascular access port and peripheral catheter placement and maintenance

A vascular access port (VAP, CompanionPort, CP 202 K, Norfolk Vet Products, Skokie, Illinois) was surgically placed under general anesthesia into the jugular vein of each cat at least 3 months before beginning the experiment. Vascular access port patency was maintained by weekly heparinized saline (10 U/mL) irrigation followed by a 0.5 mL (100 U/mL) heparin lock injection, which was aspirated and discarded before sample collection. The night before each experiment, a peripheral IV catheter (3/400 22-24ga, Terumo Survet Surflo ETFE, Ontario, Canada) was placed in a cephalic vein and removed at the end of the procedure. This cephalic catheter was used exclusively for IV infusions. For placement of cephalic catheters, cats were sedated using dexmedetomidine (1-5 μg/kg, IV). Sedation was reversed using a dexmedetomidine-equivalent volume of IM atipamezole (25-50 μg/ kg) and the cats monitored until fully recovered from sedation.

Hyperinsulinemic-hypoglycemic clamps

Controlled hypoglycemia (BG targets of 60 and 45 mg/dL, with 45 min at each target) was achieved using a modification of the hyperinsulinemic-hypoglycemic clamp protocol previously described.[14] In brief, insulin was infused at a costant rate (0.30 U/Kg/hr in one cat and 0.15 U/Kg/hr in 6 cats) and dextrose was infused at a variable rate while measuring blood glucose concentrations every 5 minutes and adjusting the dextrose infusion rate so that target glycemia levels are achived. Additional blood sampling was performed at baseline and at each clamped BG as part of a separate study in which counter-regulatory hormones were measured. Less than 24 mL of blood was drawn from each cat to account for these 13 samples (1.6 mL each) and all BG samples (0.05 mL each). This total volume accounts for ≤7% of total blood volume and therefore was deemed unlikely to affect the results of the study.

Accuracy of FSL3

The accuracy of the FSL3 was assessed by comparison to a veterinary portable blood glucose meter (vPBGM; AlphaTrak2, Blood Glucose Monitoring System, Zoetis, Parsippany, New Jersey) previously validated for use in cats with a BG range 20-750 mg/dL and intra-assay coefficient of variation of 3.8%.[16] In order to compare IG measured with FSL3 to the BG obtained with vPBGM, paired samples were collected. The FSL3 sensor (Abbott Laboratories Ltd, Chicago, Illinois) was placed, as previously described,[11] at least 1 hour before each procedure.[11] Interstitial glucose concentration was recorded using a smartphone connected to the FSL3 at the same time as each BG was measured by the vPBGM, and both were recorded as paired values. Glucose and insulin were infused exclusively via a peripheral cephalic IV catheter while all blood sampling was performed only via the jugular VAP.

Data Analysis

Data were first analyzed from all time points in which concurrent measurement of BG and IG were available. Analysis was repeated on the data subset limited to stable BG to account for blood-interstitium lag time.[11,12] Stable glycemia was defined as a change in BG of ≤1 mg/dL/min over 10 minutes preceding sample acquisition. Average absolute change first was calculated between 2 consecutive BG measurements 5 minutes apart (using the formula: [BG(t_i) BG(t_{i-5})]/5 in which *ti* is time point *I* and t_{i-5} is the time point preceding it). Average change over 10 minutes then was calculated by averaging the rate of change in the previous two 5-minute periods. If a 5 minute interim data point was missing, the rate of change was averaged directly between two readings 10 minutes apart ([BG(t_i) BG(t_{i-10})]/10). Such was the case in a single IG reading per cat, for a total of 8 data points throughout the entire data set.

Data were assessed for normal distribution using the Shapiro-Wilk test. The correlation between BG and IG was assessed using Pearson's r test and difference between glycemic groups compared using an analysis of variance (ANOVA) with Dunnett correction for multiple comparisons (with the 91-120 mg/dL glycemic range as control). Data homogeneity of variance was verified using Brown-Forsythe and Bartlett's tests. Proportional bias between BG and IG measurements was assessed using the Bland-Altman test. Accuracy was assessed according to ISO 15197:2013 guidelines, with

acceptable analytical accuracy defined as 95% of IG results being within 15 mg/dL (when BG ≤100 mg/dL) or 15% (when BG >100 mg/dL) of paired BG, and clinical accuracy as ≥99% of IG falling in zones A or B of the Parkes Error Grid analysis as formulated most strictly for people with Type I DM.[17] Statistical significance was set at $P < .05$.

RESULTS

A total of 474 paired BG and IG data points were recorded from 8 purpose-bred cats during periods of hypoglycemia and euglycemia (range, 26-164 mg/dL). Of those paired values, 324/474 (68%) were in the hypoglycemic range (BG <70 mg/dL). During periods of both stable and unstable glycemia (n = 474), BG and IG correlated moderately (r = 0.73, 95% confidence interval [CI], 0.69-0.77; P < .0001, Figure 1). In the Parkes Error Grid analysis, 99% of IG results fell in zones A and B (427 in zone A [90%], 46 in zone B [9%]), and 1% (4) of values in zone C (Figure 2). In total, 375/474 (79%) of all IG results were within 15 mg/dL (when BG ≤ 100 mg/dL) or 15% (when BG > 100 mg/dL) of their paired BG results. Overall bias between BG and IG was 0.9 ± 14.2 (95% CI, -26.9 to 28.7) mg/dL (Figure 3).

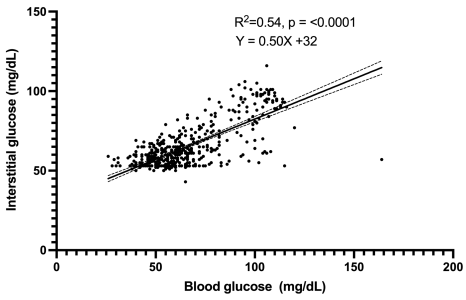

Figure 1: Correlation between blood glucose (BG) and interstitial glucose (IG) concentrations in healthy cats (n = 8) in hypo- and euglycemia at all rates of glycemic change. The solid line represents best fit with dashed lines representing the 95% CI of the best fit.

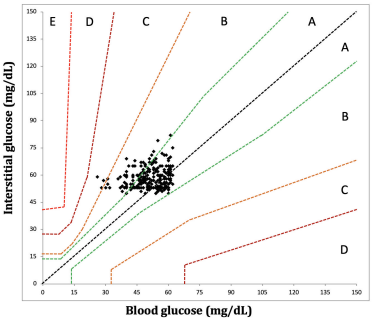

Figure 2: Parkes Error Grid analysis exhibiting excellent clinical accuracy of FSL3 at hypo- and euglycemia in healthy cats (n = 8) at all rates of glycemic change. The 99% of data points fall within zone A (n = 427, 90%) or B (n = 46, 9%).

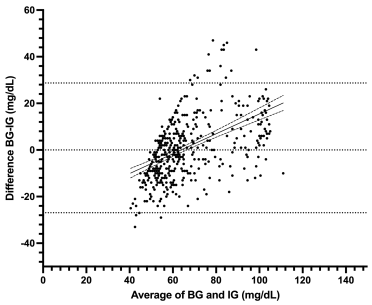

Figure 3: Bland Altman plot of agreement between blood glucose (BG) and interstitial glucose (IG) concentrations in hypo- and euglycemia in healthy cats (n = 8) at all rates of glycemic change. The middle, solid line represents best fit with dashed lines representing the 95% CI of the best fit.

Of the entire data set of 474 pairs, 301 paired values occurred during periods of stable glycemia (≤1 mg/dL/min change in BG over 10 minutes), including 218/301(72%) values in the hypoglycemic range (BG <70 mg/dL). In this subset, BG and IG correlated strongly (r = 0.86; 95% CI, 0.83-0.89; P < 0.0001; Figure 4) and 100% of IG results were in zones A and B of the Parkes Error Grid. Of these, 272 pairs were in zone A (90%) and 29 in zone B (10%; Figure 5). In total, 261/301 (87%) of all IG results were within 15 mg/dL (when BG ≤ 100 mg/dL) or 15% (when BG > 100 mg/dL) of their paired BG results. Overall bias between BG and IG was -0.07 ± 11.1 (95% CI, -21.9 to 21.8) mg/dL (Figure 6).

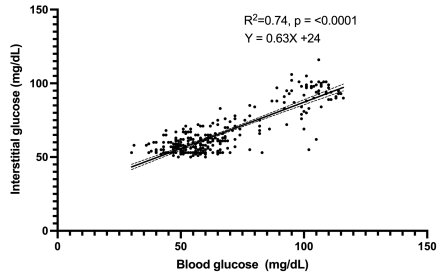

Figure 4: Correlation between blood glucose (BG) and interstitial glucose (IG) concentrations in healthy cats (n = 8) in hypo- and euglycemia during stable BG. The middle, solid line represents best fit with dashed lines representing the 95% CI of the best fit.

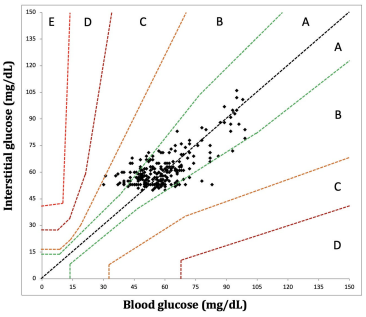

Figure 5: Parkes Error Grid analysis exhibiting excellent clinical accuracy of FSL3 at hypo- and euglycemia in healthy cats (n = 8) during stable blood glucose (BG) concentrations. All data points fall within zone A (n = 272, 90%) or B (n = 29, 10%).

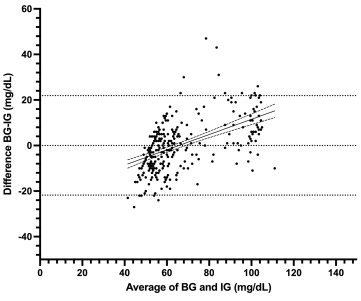

Figure 6: Bland Altman plot of agreement between blood glucose (BG) and interstitial glucose (IG) concentrations in hypo- and euglycemia in healthy cats (n = 8) during stable BG. The solid line represents best fit with dashed lines representing the 95% CI of the best fit.

Interstitial glucose concentration underestimated BG in euglycemia and mild hypoglycemia by a mean of 11.7 ± 11.2 mg/dL in the 91 to 120mg/dL range (n=59), 5.5 ± 9.1 mg/dL in the 66 to 90 mg/dL range (n = 43), and 1.5 ± 6.0 mg/dL in the 56 to 65 mg/dL range (n = 77; Figure 7). Interstitial glucose concentration instead overestimated BG by a mean of 6.3 ± 5.7 mg/dL in the 46 to 55 mg/dL range (n = 95) and 15.7 ± 5.6 mg/dL in the <45 mg/dL range (n = 27; Figure 7). Overt signs of neuroglycopenia were not observed. Adverse events were characterized by 5 episodes of vomiting (from collective data at all rates of glycemic change). One episode occurred during severe hypoglycemia (40 mg/dL), 2 during marked hypoglycemia (52-57 mg/dL), and 2 during moderate hypoglycemia (60 mg/dL).

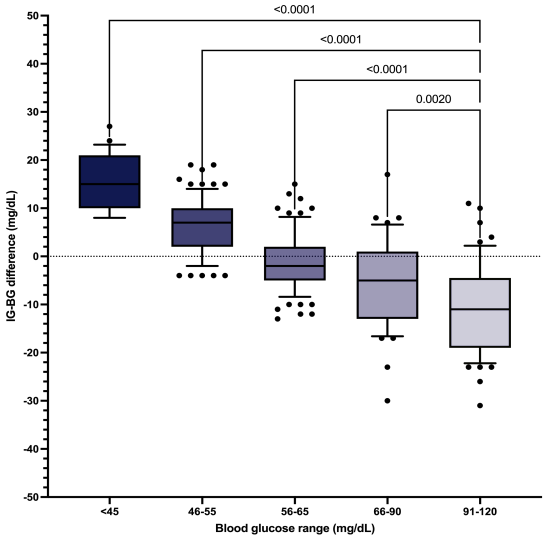

Figure 7: Difference between interstitial glucose (IG) and blood glucose (BG) concentrations, stratified based on BG range in healthy cats (n = 8) during stable BG. Central horizontal lines represent median values, boxes represent quartiles, whiskers represent 10% to 90% percentiles.

DISCUSSION

The results of this study showed a good clinical accuracy of FSL3 during hypo- and euglycemia. The use of CGMS represents a paradigm shift in the management of veterinary diabetic patients.[2] Several studies have described the accuracy of previous generation of FSL in cats,[4,10-12,14] but none have evaluated the performance of FSL3. The accuracy of FSL3 in cats during hypoglycemia and euglycemia is reported here with a total of 474 paired BG-IG measurements, 324 of which were in the hypoglycemic range (BG <70 mg/dL), including 218 paired values recorded during periods of stable glycemia. A strong positive correlation between IG and BG was observed during periods of stable glycemia. However, when considering the entire dataset, that includes both stable and unstable BG, we found a moderate correlation. Our results are similar to a previous study evaluating the FSL2 in hypo- and euglycemic cats.[14] Direct comparison with other studies is limited by difference in hypoglycemic sample size, method of reference glucose measurement (vPBGM vs hexokinase-based laboratory assays), study populations (client-owned diabetic vs purpose-bred cats), and differences in sampling environments and frequencies (home-based, hospital-based, or controlled research laboratory settings).[4,10-12]

Similar to previous veterinary CGM studies utilizing ISO 15197:2013 guidelines, standards for clinical (but not analytical) accuracy were met in our study. Only 79% of the data points met the analytical accuracy criteria outlined by the ISO 15197:2013 standards. Our results are comparable to previous studies in cats, with a reported analytical accuracy that ranged from 42% to 73%.[10-12,14] Parkes EGA demonstrated strong clinical accuracy, with 100% and 99% of values classified within zones A and B during periods of stable and unstable glycemia, respectively. The ISO 15197:2013 guidelines require that PBGM measurements be compared to a standardized reference method. However, these standards

are tailored for comparisons within a single compartment, typically blood, and might not be fully applicable for cross-compartmental comparisons (i.e., between blood and interstitial fluid), due to the physiological differences between these compartments.

Analytical accuracy is not expected for CGMS and generally is not met because IG and BG measure glucose in different compartments.[10-12,14] Given the absence of well-established criteria for assessing glucose measurement accuracy in interstitial fluid, the ISO standards for PBGMs offer a useful proxy, helping to identify CGMS devices that closely adhere to accuracy criteria and that are not dangerous for the individual's health. With these limitations in mind, the FSL3 can be considered suitable for clinical use in cats, demonstrating performance similar to the FSL2 in the euglycemic and hypoglycemic ranges. Despite the good clinical accuracy of earlier FSL models, premature sensor detachment remains one of the most commonly reported complications in diabetic cats, with median sensor activity ranging from 5 to 10 days.[4,10-12] Additionally, in a recent survey, one of the most significant concerns expressed by owners of diabetic pet, particularly those of diabetic cats, was the reduced FSL lifespan.[23] The smaller size of the FSL3 might improve both tolerability and adherence to long-term CGMS use in feline patients. In our study, it was not possible to assess the duration and tolerability of the sensor in the experimental setting; therefore, further studies are needed in diabetic cats.

Our data suggest a small and proportional bias in IG values in the hypoglycemic range. As previously reported in cats with FSL2,[14] IG measured by FSL3 tends to underestimate BG in the euglycemic range in cats, with the difference decreasing as BG levels decrease. However, in cases of marked hypoglycemia, IG tends to overestimate BG in healthy cats. Based on our findings, IG readings in the severely hypoglycemic range (<45 mg/dL) should be approached with caution and interpreted as potentially representing BG at an equal or lower concentration. Hypoglycemia is a major limiting factor in the management of DM in patients receiving insulin therapy. In surveys that investigated quality of life of owners of diabetic pets, as well as perceived quality of life of their diabetic pets, owners' fears of hypoglycemia had one of the largest negative impacts on their quality of life.[8,9] The CGMS improves the detection of hypoglycemic episodes[3] and might reduce the incidence of clinical hypoglycemia when used for insulin dose titration.[18] In our study, the experimentally induced hypoglycemia offered a valuable opportunity to ethically and efficiently collect a large number of IG data within this glycemic range. In a clinical context, discrepancies between BG and IG concentrations can partially be attributed to the time necessary for glucose concentrations to equilibrate between the blood and interstitial fluid. By maintaining a stable BG using the glucose clamp technique, we were able to more precisely evaluate the bias in IG measurement relative to BG, minimizing the confounding effects of the lag time required for equilibrium between the 2 compartments. This lag in detecting changes in glucose concentrations likely results from both biological and technical considerations associated with measurement of IG. First, there is a temporal gap between fluctuations in BG and the time required for these changes to be reflected in the interstitial fluid. This is influenced by factors such as glucose transport across endothelial barriers and the concentration gradient.[11] Second, the diffusion distance from blood vessels to the sensor adds to lag time, as well as algorithmic delay in reporting the IG. Previously reported lag times vary widely based on the method used to induce a change in BG, whether BG is increasing or decreasing, the CGMS used, and the definition of lag time.[11,19] In cats, the rapid administration of high-dose intravenous glucose (0.5 g/kg) leads to a delay of 5 to 15 minutes before an initial rise in IG concentrations are observed, and 30 to 45 minutes until peak IG levels are observed.[11] This delay is further extended in patients with reduced interstitial tissue perfusion, such as elderly or dehydrated animals,[11,20] as well as when BG fluctuations are rapid and of significant magnitude.[21] The cats included in our study were clinically normohydrated and within the young-adult age range. Hence, BG-IG lag time obtained in dehydrated and older cats may require additional evaluations. However, age and hydration status are not expected to affect the accuracy of the system in stable conditions.

In diabetic cats, BG might vary substantially both throughout the day and across consecutive days.[22] There are several factors contributing to BG variability, including inconsistent insulin absorption and degradation, variable amount of residual β cells, technical issues with insulin administration, concurrent illnesses, and varying levels of stress, among others.[22] In contrast, in healthy cats, BG exhibit minimal variation from day-to-day. In our study, the magnitude of BG changes was confined to a narrow hypoglycemic range, and sympathetic responses that could lead to unpredictable and rapid BG fluctuations were minimized through restraint-free handling and acclimatization to study personnel and laboratory conditions. Therefore, in clinical practice, the FSL3 is not optimal for estimating BG at the moment of its measurement. However, it is exactly because of substantial glucose variability in diabetics that clinically, measuring IG in a continuous manner is superior to measuring BG in predicting future BG's and making treatment decisions. Therefore, with the exception of confirming specific measurements, for example, when a diagnosis of neuroglycopenia needs to be confirmed, there is no advantage to confirming IG readings with BG, even when the rate of IG change is high.

Vomiting was the only adverse effect observed in response to hypoglycemia in our population of fasted, healthy cats, consistent with findings from our previous study.[14] In that study, vomiting did not appear to correlate with the severity of hypoglycemia, as it was observed at both moderate (60 mg/dL) and severe (40 mg/dL) hypoglycemia. The occurrence of vomiting could be considered a warning sign prompting caregivers to check their cat's BG levels. However, due to the experimental nature of this study, definitive conclusions cannot be drawn, and the relationship between vomiting and hypoglycemia in cats requires further investigation.

Limitations of this study include the use of only non-diabetic cats with experimentally induced hypoglycemia for data collection. Hypoglycemia was achieved through regular insulin infusion, leading to rapid fluctuations in BG concentrations, which may not fully replicate the glycemic patterns seen with intermediate- or long-acting insulin formulations typically used in a clinical setting. Although the physiological mechanisms underlying the discrepancy between BG and IG are likely consistent regardless of the cause of hypoglycemia, further research is essential to validate these findings in diabetic cats and to assess the accuracy of the device within the hyperglycemic range.

In conclusion, the FSL3 provides clinically accurate measurements in the euglycemic and hypoglycemic ranges. Clinical interventions prompted by IG measurements within the hypoglycemic range can have significant consequences. Therefore, recognizing the proportional glycemic-dependent bias associated with FSL3 IG allows clinicians to enhance the safety of its application. Clinicians should be cautioned that although the FSL3 tends to underestimate BG in most of the euglycemic range in cats, it may overestimate BG in hypoglycemic ranges <55 mg/dL. Interstitial glucose readings in the severely hypoglycemic range should be approached with caution and it might be advisable to assess BG using a validated vPBGM to confirm the FSL3 results. The smaller size of FLS3 could enhance tolerability and extend the sensor lifespan, facilitating the monitoring of diabetic cats. Further studies are needed to assess the performance and tolerability of the FSL3 in diabetic cats.

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3.7 | The usefulness of different FreeStyle Libre-derived metrics in assessing glycemic control in diabetic dogs

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ABSTRACT

Background

Flash glucose monitoring system (FGMS, FreeStyle Libre®) is nowadays routinely used in diabetic dogs (DD) and a few studies have demonstrated its accuracy and clinical utility. However, successful utilization of FGMS data in routine clinical practice remains relatively low because there is a lack of agreement regarding the interpretation of FGMS data.

Objectives

This study aims to assess the utility of different metrics readily available with the use of FGMS to monitor DD.

Methods

Fourteen DD on insulin treatment (12 porcine lente insulin, 2 Neutral Protamine Hagedorn insulin) were retrospectively enrolled in the study. A single evaluation for each patient was included. All dogs were monitored with FGMS, and data were collected after at least 7 days of continuous glucose detection. The glycemic control was classified according to the ESVE ALIVE clinical score (CS) that takes into account the stability of body weight, presence of polyuria/polydipsia, activity/attitude, and appetite. The clinical score range from 0 (optimal) to 12 (poor). The following metrics were evaluated: percent time in range (percentage of time glucose within 70-250 mg/dL;TIR%), percent time above range (percentage of time glucose above 250 mg/dL;TAR%), percent time below range (percentage of time glucose below 70 mg/dL;TBR%), median glucose (MG), percent coefficient of variation (CV%). Correlations between CS and TIR%, TAR%, TBR%, CV%, and MG were evaluated. Moreover, the correlation between CV% and MG was assessed. Mann-Whitney test was used to compare CV% in dogs with and without concurrent diseases, as well as in dogs with and without clinical hypoglycemia.

Results

TIR% , TAR% and TBR% were significantly correlated with the CS (rs=-0.79,P=0.001; rs=0.79,P=0.001; and rs=-056,P=0.04; respectively). Moreover, a significant correlation between MG and CS was found (rs=0.79, P=0.001). CV% was inversely correlated with MG and CS (rs=-0.90, P<0.0001; rs=-0.78, P=0.002;respectively). CV% was 48.7% and 32.8% in dogs with clinical hypoglycemia and dogs that did not experience clinical hypoglycemia, respectively (P=0.10). Further, CV% was higher in dogs with concurrent diseases compared to dogs without concurrent diseases (37.6 vs 27.5%), although the difference was not significant (P=0.75).

Conclusions and clinical importance

This is the first study evaluating FGMS-derived metrics in DD. The strong correlation between TIR%, TAR%, TBR%, MG, and CS suggests the potential clinical usefulness of these metrics for monitoring DD. The CV% does not seem to reflect the short-term glycemic control. Although not significant, CV% seems to be higher in dogs with clinical hypoglycemia and with concurrent diseases.

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3.8 | Monitoring of Diabetes Mellitus Using the Flash Glucose Monitoring System: The Owners' Point of View

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ABSTRACT

Flash glucose monitoring system (FGMS) has recently become one of the most common monitor-ing methods in dogs and cats with diabetes mellitus. The aim of this study was to evaluate the impact of FGMS on diabetic pet owners (DPOs) quality of life. Fifty DPOs were asked to answer 30 questions survey. More than 80% of DPOs considered FGMS easier to use, less stressful and painful for the animal compared to blood glucose curves (BGCs). Overall, 92% of DPOs reported that their pet had better diabetes control since using FGMS. The most challenging aspects of using the FGMS were ensuring proper sensor fixation during the wearing period (47%), preventing premature detachment (40%) and purchasing the sensor (34%). Moreover, 36% of DPOs reported that the device cost was difficult to afford in the long term. Comparing dogs and cats, a signifi-cantly higher number of dogs' owners retained FGMS well-tolerated (79% vs 40%) and less in-vasive than BGCs (79% vs 43%) and easier to maintain in situ (76% vs 43%). In conclusion, FGMS is considered by DPOs easy to use, less stressful compared to BGCs while enabling better glyce-mic control. Nevertheless, costs related to its long-term use might be difficult to sustain.

INTRODUCTION

Dogs and cats with diabetes mellitus (DM) are frequently treated with exogenous insulin and a specific diet and require regular monitoring to ensure appropriate dosing.[1] In recent years, glucose monitoring has been revolutionized by the advent of continuous glucose monitoring systems (CGMSs). According to the author's experience, these systems are progressively replacing the use of blood glucose curves (BGCs) and are nowadays one of the most widely used monitoring methods for diabetic pets. The FreeStyle Libre® flash glucose monitoring system (FGMS, Abbott Laboratories Ltd., Chicago, IL, USA) is a commonly used CGMS, thanks to its easy-to-use and long sensor lifespan. This device measures interstitial glucose (IG) concentration, which correlates well with blood glucose (BG).[2,3] However, a lag time occurs between changes in BG and IG, and the latter also is affected by local factors specific to the tissue in which it is measured.[4,5] In dogs, the FGMS provides detailed IG profiles, allowing for the more accurate detection of nadir and hypoglycemic episodes as compared to BGCs generated by a portable blood glucose meter (PBGM).[6] It also allows for the detailed identification of the glycemic excursions occurring throughout the day or on different days.[7] In veterinary medicine, it is generally accepted that owner compliance is essential for successfully treating DM.[8] The disease and the treatment commitments are likely to have a considerable impact on owners' daily routines and quality of life (QoL) and might represent a significant temporal, financial, and emotional burden. In support of this, a recent study showed that more than 30% of diabetic pet owners (DPOs) euthanize their pets due to the negative impact of DM management on their lifestyle.[8] For this reason, it is crucial to consider the impact of DM management and of the different monitoring methods on the QoL of DPOs. In veterinary medicine, the impact of a particular monitoring method on the QoL of DPOs has rarely been investigated. In one study, the use of home blood glucose monitoring was associated with positive changes in the QoL parameters of cats and their owners and significant glycemic improvements.[9] In two recent studies, DPOs were asked to complete a questionnaire regarding their experience with the FGMS,[10,11] while a third one has evaluated owner satisfaction with the use of an FGMS through a questionnaire containing 16 yes-or-no questions.[12] The FGMS was considered to be easy to use by DPOs and provided great satisfaction.[10–12] Moreover, in human medicine, the use of an FGMS positively influences the QoL of diabetic patients since it significantly reduces the risk of hypoglycemic episodes, which negatively impact the QoL of diabetic patients.[13] Despite the fact that the convenience of the use of an FGMS has been sporadically addressed in previous canine and feline studies, no studies have evaluated the impact on the QoL associated with the use of an FGMS on DPOs. Therefore, the aim of this study was to investigate the impact of an FGMS on diabetic pet owners' QoL and the satisfaction related to its usability.

MATERIALS AND METHODS

Participants and Questionnaire

Diabetic pet owners whose animals were admitted to the Veterinary Teaching Hospital of the University of Bologna from July 2021 to September 2022 were asked to complete an online survey (Google Form, https://forms.gle/GHT2y6J1FTzKmwaX6, accessed on 1 December 2022). Owners were considered to be eligible for inclusion in the study if they had used at least one FGMS. The survey was made up of thirty questions, including multiple-choice (M) questions (5/30), single-option questions (S) (20/30), and free-text statements (F) (5/30). The survey was divided into three categories: (1) questions related to the technical use of the FGMS (Table 1), (2) a comparison between the use of an FGMS and the generation of BGCs (Table 2), and (3) the impact of an FGMS on diabetic pets and the QoL of DPOs (Table 3).

Table 1. Questions related to the technical use of the flash glucose monitoring system (FGMS).

Question	Answer Form
When did you start using the FGMS monitoring method?	F
How many devices has your pet used so far?	F
How many devices could not be used due to operating problems or early detachment (within 24 h of insertion)?	F
Who proposed the FGMS to you?	S
Do you think that the information provided has been useful to understand and use the device?	S
Who applies the sensor?	S
How is the FGMS fixed to the skin?	S
Once applied, is the sensor protected by a bandage?	S
In which body area is the FGMS usually applied?	M
Is the application area of the sensor always the same?	S
How are the glucose data of your animal transmitted to the vet?	S
How long after your pet's diagnosis of diabetes did you start using the FGMS?	F

Table 2. Questions related to the comparison between the use of flash glucose monitoring system (FGMS) and the generation of blood glucose curves (BGCs).

Question	Answer Form
What are the main advantages related to the use of the sensor?	M
Can you explain how your pet's blood glucose was monitored [if you haven't used the FGMS since the onset of the disease]?	M
Compared to BGCs, do you think that FGMS is an easier monitoring method?	S
Compared to BGCs, do you think that FGMS provides better glycemic control?	S
Compared to BGCs, do you think that the FGMS is less stressful?	S

Table 3. Questions related to the impact of a flash glucose monitoring system (FGMS) on the quality of life (QoL) of diabetic pets and diabetic pet owners (DPOs).

Question	Answer Form
What are the main drawbacks of using the sensor?	M
Do you think an FGMS allows better glycemic control?	S
Do you think an FGMS is well tolerated by your pet?	S
Does an FGMS have a negative impact on your QoL?	S
How many times a day do you scan the sensor?	S
How often do you apply the FGMS to your diabetic pet?	S
How do you feel about continuously accessing your pet glucose values?	S
What do you think about costs related to the use of FGMS?	S
Are you currently using an FGMS on your diabetic pet?	S
Will you use an FGMS again?	S
Why? [If you gave a negative answer to the previous question]	M
Would you recommend the FGMS to other DPOs?	S
Express you opinion	F

Flash glucose monitoring system

The FGMS used by the owners was FreeStyle Libre Abbott®. This device is available online via the manufacturer's official website. Its technical features and the application procedures have been described in previous studies.[5,14] Scanning using the sensor needs to be carried out at least every 8 h; it automatically records the IG values every fifteen minutes. The IG trends are transferred from the sensor to a reader when the user brings the handheld reader into close proximity to the sensor. The FreeStyle Libre Link® mobile app can be used as an alternative to the reader. The reader stores the data for 90 days, and, if the scans are performed using the FreeStyle Libre Link® app (software version 2.8.1.6120, Abbott Laboratories Ltd., Chicago, IL, USA), the glucose values are automatically uploaded to Libreview® (https://www.libreview.com, accessed on 1 December 2022) when the phone is connected to the Internet. Libreview® is a free, secure, cloud-based diabetes management system provided by Abbott. The system generates summary glucose

reports from the uploaded sensor data, readily available for consultation by healthcare providers. The report provides a graphical trace of the glucose values of a 24 h period, allowing access to previous glucose data.

Statistical Analysis

Statistical analysis was carried out using a commercially available software program (MedCalc Software Ltd., Ostend, Belgium, version 20.121). Owing to the small number of cases, the continuous variables were considered to be non-parametric, and descriptive statistics were reported as a median (minimum–maximum). The categorical variables were reported as frequencies, proportions, or percentages. The differences between dog and cat DPOs regarding the tolerability of the sensor, impact on glycemic control, stress degree related to the monitoring methods (FGMS vs. BGCs), and problems related to premature sensor detachment were compared using the Fisher's exact test. Values of $p < 0.05$ were considered significant.

RESULTS

Technical Use of the FGMS

Fifty DPOs were enrolled in the study. Of them, 29/50 (58%) were dog owners and 21/50 (42%) were cat owners. The median (range) number of FGMSs used by each DPO was 4 (1–10). The number of FGMS used by each DPO was 1 in 5 cases, 2–5 in 29 cases, 6–9 in 7 cases, and 10 or more in 9 cases. Forty-two percent of DPOs reported a premature end of the sensor within 24 h of placement due to early detachment or malfunctioning. Of them, 24/21 (76%) were dog owners and 16/21 (76%) were cat owners. Among DPOs who used only one sensor, no one reported an early detachment on the first day of use.

The use of the FGMS was proposed to the DPOs by a referral center (31/50, 62%), was recommended by the primary care veterinarian (10/50, 20%), or was discovered by the owners themselves (9/50, 18%). Forty-three percent of the DPOs understood how to use the sensor, based only on the instructions provided by the veterinarian. In contrast, 14% of them (28/50) had to find more information on the Internet regarding its use (e.g., sensor manufacturer's website, Youtube® videos, and online forums). In 58% (29/50) of cases, the FGMS was placed exclusively by the veterinarian, while, in 42% (21/50) of the DPOs (68% of dog owners, 14/21; and 32% of cat owners, 7/21), it was placed by the owner. A total of 68% (34/50) of DPOs (70% cat owners, 24/34; and 30% dog owners, 10/34) reported that additional glue was necessary to better fix the sensor onto the skin. Of these, 26% (9/34) used a liquid medical adhesive, and 74% (25/34) used a cyanoacrylate glue. Moreover, in 88% of cases (44/50), the sensor was protected with an additional bandage (cotton and elastic bandage). The sensor lifespan reported by the manufacturer (14 days) was reached in 20% of cases.

The most widely used application area of the sensor was the dorsal aspect of the neck (78% of cases, 39/50), followed by the dorsum (18% of cases, 9/50). In one case, the sensor was applied on the shoulder blade region, and in another case, it was applied on the lumbar–sacral region. Twenty-six percent (13/50) of the DPOs changed the application area for each new sensor, by rotating between their favorite application areas. Forty-nine of the fifty DPOs (98%) used the specific FGMS mobile app as a sensor reader, while only one DPO (2%) used the handheld portable reader. The glucose values obtained using the sensor were transmitted to the veterinarian by means of the Libreview® data-sharing mode in 66% of cases (33/50). The remaining DPOs shared glucose values and information regarding animal health by creating Excel files or paper notes. Twenty-five DPOs (50%) began using an FGMS within three months from the DM diagnosis, while the remaining DPOs started using it three months after (up to two years) the DM diagnosis.

Comparison between an FGMS and BGCs

Before using an FGMS, all the diabetic pets were monitored with BGCs carried out at home or in the hospital. In particular, all the DPOs included experienced home monitoring by performing at least one BGC at home.

When comparing the use of an FGMS with a BGC, we noted that 85% (43/50) of the DPOs believed that the FGMS was easier to use than a PBGM. In addition, in 82% (41/50) of cases, the FGMS was considered less stressful and painful than a BGC. As shown by Figure 1, 79% of dog owners (23/29) considered the FGMS application to be less invasive than carrying out a BGC. In contrast, 57% of cat owners (12/21) consider it as invasive as carrying out a BGC; a significant difference was found between canine and feline DPOs (p = 0.01).

In the owner's opinion, the major advantages of using the FGMS were less stress for the animal than carrying out a BGC at home or in the hospital (40/50, 79%), the possibility of obtaining more information on the glucose trend with less effort (34/50, 67%), the low invasiveness and better comfort for the animal (32/50, 64%), the ease of use (29/50, 58%) and the reliability of the results provided by the FGMS (23/50, 45%). The long-term use of the device was considered to be too expensive in 36% of cases (18/50), difficult to afford in 14% of cases (7/50) and affordable in 50% of cases (25/50) Overall, 92% of the DPOs (46/50) believed their pet had better glycemic control since using the FGMS as a monitoring method. No differences were found between dog and cat DPOs (Figure 2; P=0.29).

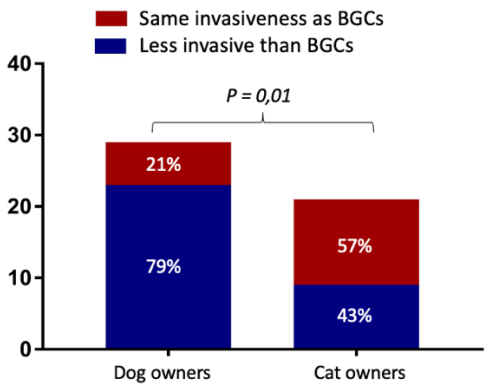

Figure 1. Comparison of dog and cat owners' points of view regarding the invasiveness of the flash glucose monitoring system (FGMS) when compared to blood glucose curves (BGCs).

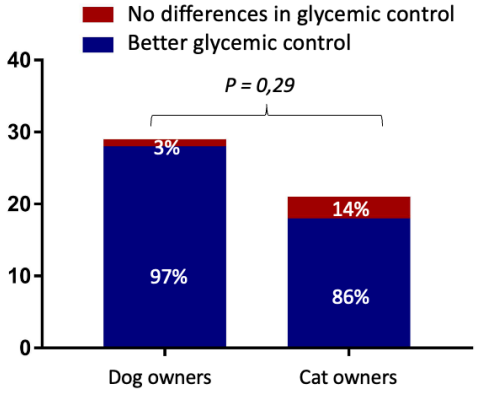

Figure 2. Comparison of dog and cat owners' points of view regarding glycemic control of the flash glucose monitoring system (FGMS) when compared to blood glucose curves (BGCs).

Impact of an FGMS on diabetic pets' and DPOs' quality of life

The most challenging and stressful aspects of using the sensor were ensuring ad-equate fixation during the operating period (24/50, 47%), preventing self-removal by scratching or licking (20/50, 40%) and the purchase of the sensor online (17/50, 34%). In particular, premature sensor detachment was a concern described by 57% (12/21) of cat DPOs and by 24% (7/29) of dog DPOs (Figure 3 and P=0.02). In addition, 60% (13/21) of cat DPOs reported that the sensor was not well tolerated, and a significant difference was found when compared to dog DPOs (Figure 4).

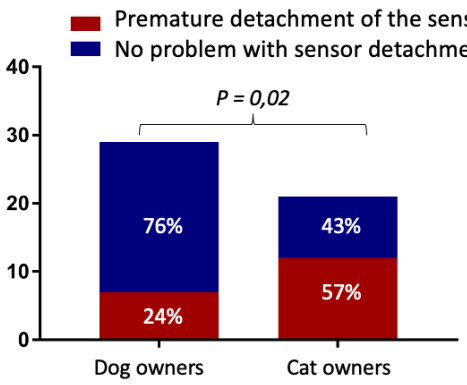

Figure 3. Comparison of dog and cat owners' points of view regarding problems related to premature sensor detachment.

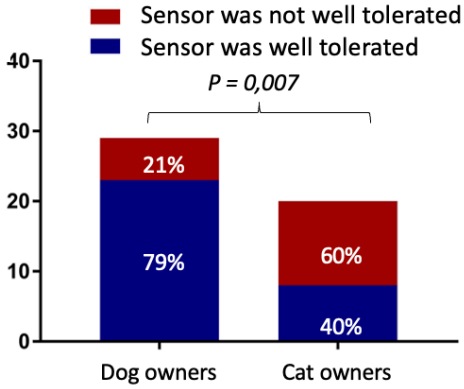

Figure 4. Comparison of dog and cat owners' points of view regarding tolerability of the sensor.

Mild-to-moderate dermatological complications after sensor removal were reported in 18% of cases (9/50). Thirty-five of the fifty DPOs (70%) stated that using an FGMS had no negative impact on their QoL. Forty-four percent of the DPOs (22/50) felt safer replacing the FGMS whenever the previous sensor stopped working. The continuous access to the glucose data generated a sense of reassurance (92%, 46/50) or increased anxiety (8%, 4/50). The number of daily scans carried out by the DPOs is shown in Figure 5.

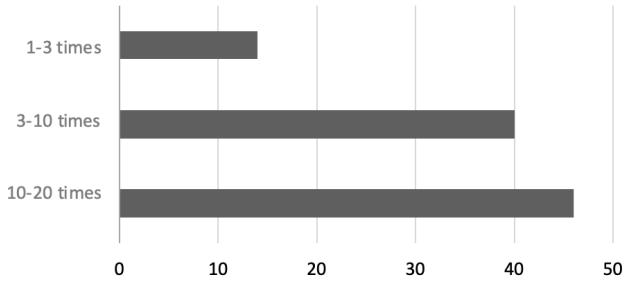

Figure 5. Number of sensor scans per day carried out by dog and cat owners when using the flash glucose monitoring system.

At the time of filling out the survey, 29/50 DPOs (58%) were still using the FGMS on their diabetic pet, and 84% of them (42/50) would continue to use it in the future. The remaining 16% of the DPOs (8/50) would not continue using the FGMS owing to its elevated cost (32/50, 64%), the difficulty of buying it (9/50, 18%), and excessive stress for the animal (3/50, 6%). Forty-seven of the fifty (94%) DPOs would recommend the FGMS to other owners of diabetic pets.

DISCUSSION

The FGMS is an increasingly widespread monitoring method for DM in veterinary patients. The aim of this study was to investigate the impact of an FGMS on DPOs' QoL and the satisfaction related to its usability. According to the present results, using an FGMS as a monitoring tool provided better glycemic control than BGCs. Moreover, continuous access to the glucose data generated a sense of reassurance in the majority of the DPOs. Despite this, the main drawbacks reported by DPOs were the increased anxiety related to the possibility of having continuous access to their diabetic pet glucose values and the costs related to its use. An FGMS is designed to be worn for fourteen days. Despite this, one of the most negative aspects described by the DPOs was the reduced sensor lifespan. This was especially true in diabetic cats, and the present results are in agreement with those of previous studies.[5,10,15] In contrast, the reduced sensor lifespan was less frequently reported by dog owners. These results are in agreement with those observed in previous studies in which the maximal duration of the FGMS (14 days) was reached in about 70% of cases.[12,14] In fact, the premature detachment of the sensor represents one of the most frequent complications in diabetic cats, with a median sensor wearing time ranging from 5 to 10 days.[5,7,10,16] For this reason, in cats, to extend the sensor-wearing time, it might be advisable to additionally secure the sensor by using more glue. In the present study, approximately two-thirds of the DPOs used additional glue to extend the sensor- wearing time. This was more common among cat owners. The most used type of glue was cyanoacrylate (a multipurpose non-medical glue) due to its low cost and easy availability. Liquid medical adhesive, which is generally applied to fix dressings, patches, and some medical devices, was used in a minority of cases. Despite this, in the present study, only 20% of the sensors reached the working life of 14 days reported by the manufacturer for diabetic patients. The use of skin stitches has recently been described as a method for securing the sensor in cats.[16] In the authors' cases, skin stitches were not used, mainly due to the excessive invasiveness of the procedure and the need to perform it exclusively in the hospital.

Almost half of the DPOs (mainly dog owners) were able to apply the sensor on their own at home. This represented an important factor in reducing costs in the management of diabetic pets. Similar to recent studies, dermatologic complications associated with the use of FGMS were mild and self-limiting.[6,10,12,17] However, severe allergic contact dermatitis, caused by the adhesive part of the sensor, has been reported in diabetic people.[18]

In the present study, the most common application site was the dorsal aspect of the neck. This is the area recommended by the authors' veterinary hospital since it was the most commonly used location in validation studies.[5,6,14] Moreover, this area allows for an additional bandage (applied by almost 90% of the DPOs). The dorsum was the second most common application site, followed by the thoracic wall. In veterinary medicine, two studies have investigated the effect of the sensor location on the performance of another CGMS (Guardian Real-Time). In dogs, the IG measured in the chest site had the best correlation with blood glucose concentration as compared to the neck site; however, the sensor had the shortest lifespan.[19] Conversely, in cats, the dorsal neck area provided superior results in terms of accuracy when compared with the lateral chest-wall and knee fold.[20] Unfortunately, there are no data available as to whether different application sites could influence the performance of the FGMS in dogs and cats.

All the glucose values obtained during the sensor-wearing period were transmitted by DPOs to the attending veterinarian for his evaluation to aid in therapeutic decisions. The most widely used data-sharing mode was Libreview®, which is a cloud-based diabetes management system in which the glucose readings from the FGMS can be uploaded and shared with the healthcare professional team. This monitoring method allows monitoring the glucose trend by forming a graphical trace of glucose values over a 24 h period and having access to previous glucose data. Moreover, it provides some metrics, such as the average glucose, coefficient of variation (CV), and time of glucose within/below/above range. To date, in veterinary medicine, a single study addressed one of these parameters (CV);[12] however, their practical application might increase in the future. In fact, the concept of glycemic variability is emerging in human medicine as an additional glycemic target,[21] and a few studies have started to investigate its role in veterinary.[22,23]

Several studies have described the accuracy and clinical utility of an FGMS in dogs and cats.[6,7] It has been demonstrated that an FGMS allows for more accurate identification of the glucose nadirs, post-prandial hyperglycemia, hypoglycemic episodes, and day-to-day variations in glycemic control as compared to BGCs. For this reason, the FGMS is being used more and more; therefore, it was decided to also evaluate the owners' point of view. Approximately 80% of DPOs reported that the use of an FGMS was easier, less stressful, and less painful than carrying out BGCs. This could be explained by the fact that the application of the sensor is fast and painless. Furthermore, a majority of the DPOs were able to apply the sensor themselves. For obtaining a BGC, blood sampling is required, and when the BGC is not carried out at home, the animal requires hospitalization for at least 8–10 h. In addition, the possibility of assessing continuous glucose data remotely by using the Libreview® system allows for insulin-dose adjustments, without taking the animal to the hospital. This aspect is particularly relevant for diabetic cats in which stress hyperglycemia is a common problem in the interpretation of the BGC. Nevertheless, unlike dog owners, cat owners considered the application of an FGMS to be as invasive as carrying out a BGC. This result could be explained by the lower tolerability of the sensor application and wearing by the cats. For this reason, the discomfort from wearing the sensor may be perceived by the DPOs as a sign of excessive invasiveness for the cat.

In the current study, 92% of the DPOs believed that their pet had better glycemic control since using the FGMS monitoring method. It was recently reported that, if DM is monitored using a PBGM, glucose fluctuations between blood glucose measurements might be missed, and this could result in erroneous insulin-dose recommendations.[24,25] Moreover, by monitoring glucose trends remotely, insulin-dose adjustments can be performed more frequently and probably more effectively than by carrying out BGCs. Therefore, in the authors' opinion, these advantages may result in a better

perception of glycemic control by DPOs. Nevertheless, these results might be biased by the fact that some dogs and cats were referred for sensor placement, as glucose readings were not possible or difficult to perform, and therefore DPOs asked for a different monitoring method.

Regarding the impact of an FGMS on the DPOs' QoL, 92% of cases experienced a sense of reassurance in being able to continuously know the glucose values of their diabetic pet. Moreover, 42% of DPOs apply the sensor, continuously replacing each sensor at the end of its use with a new one. In veterinary medicine, an FGMS is used as an alternative monitoring method to BGCs. Therefore, in the authors' clinical practice, they apply the sensor continuously until an optimal insulin dose is identified. Despite the fact that the majority of the DPOs felt a sense of reassurance, 8% of them reported that the chance to have continuous access to their diabetic pet's glucose values caused increased anxiety. This was highlighted by the fact that 46% of the DPOs carried out between 10 and 20 glucose readings per day, although this is not necessary for the correct functioning of the sensor. In the authors' opinion, anxiety could probably increase when DPOs detect low glucose values. However, this aspect was not evaluated in the present study.

The other major drawbacks associated with the use of the FGMS were its cost and its availability. Currently, in the authors' country, the FGMS can only be purchased online via the official website of the manufacturer. This aspect is particularly challenging for the elderly or for those who are not familiar with the use of the Internet. In fact, 34% of DPOs stated that availability was one of the most negative aspects associated with the use of the device. Based on these results, the possibility of buying the sensor not only online but also through other sellers could probably make it more usable by all types of DPOs. In addition to this, in 37% of the cases, the long-term use of the device was considered too expensive. This was in agreement with previous studies in which, despite the elevated degree of satisfaction, the cost was reported to be a main drawback.[10,12] Therefore, this seems to be a common problem in different countries. Nevertheless, despite the disadvantages reported, 70% of the DPOs reported that using an FGMS had no negative impact on their QoL; this was in agreement with previous studies in human medicine in which the continuous use of an FGMS was associated with an improved QoL in diabetic patients.[26–29] Moreover, Overend et al. reported that an FGMS had a positive impact on psychological well-being and self-esteem since patients with type 1 DM experienced more control over their BG values.[30] In total, 84% of the DPOs stated that they would continue to use the device in the future, and 94% of them would recommend it to other DPOs. These data suggest that the overall good DPO satisfaction and owner perceptions of the advantages of FGMS outweigh the disadvantages.

The present study had some limitations, including the small sample size, its retrospective nature, and the fact that the survey used was not previously validated. Another limitation of this study is that the degree of stress of the diabetic pet and the DPOs' QoL were evaluated subjectively and not through specific scores. However, the main limitation was that all the diabetic patients included were monitored at a referral center. In fact, thanks to the specialist medical staff, the DPOs were well-instructed regarding the use of the sensor and how to interpret the glucose data. This might have positively influenced the present results. For this reason, additional studies, also including diabetic pets managed by primary care veterinarians, are needed.

CONCLUSIONS

In conclusion, the FGMS was considered easy to use by the DPOs and less stressful when compared to BGCs, while enabling better glycemic control. Moreover, the possibility of having continuous access to the glucose data generated a sense of control in the DPOs. Nevertheless, the cost related to its long-term use might be difficult to sustain. Additional reported drawbacks were the availability of the sensor and the increased sense of anxiety of the DPOs. Finally, in cats, premature detachment and poor tolerability of the device are frequent concerns.

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3.9 | Clinical Use of a 180-Day Implantable Glucose Monitoring System in Dogs with Diabetes Mellitus: A Case Series

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ABSTRACT

The novel Eversense XL continuous glucose monitoring system (Senseonics, Inc., Germantown, Maryland) has recently been developed for monitoring diabetes in humans. The sensor is fully implanted and has a functional life of up to 180 days. The present study describes the use of Eversense XL in three diabetic dogs (DD) with good glycemic control managed by motivated owners. The insertion and use of the device were straightforward and well tolerated by the dogs. During the wearing period, some device-related drawbacks, such as sensor dislocation and daily calibrations, were reported. A good correlation between the glucose values measured by the Eversense XL and those obtained with two commercially available devices, previously validated for use in DD, was found ($r_s = 0.85$ and $r_s = 0.81$, respectively). The life of the sensor was 180 days in two of the DD and provided high satisfaction. This innovative device might be considered a future alternative for home glucose monitoring in DD.

INTRODUCTION

Glycemic control is a crucial aspect of the management of diabetes mellitus (DM) and is essential for the prevention of complications in both human and veterinary medicine. Continuous glucose monitoring systems (CGMSs) are frequently used in humans with diabetes, and clinical studies have shown they are effective in reducing hypoglycemia and improving glycemic control.[1–8] Thanks to their high performance, CGMSs have gained popularity among veterinarians and are increasingly being used in diabetic dogs (DD) and cats.[9–19] CGMSs measure, using a transcutaneous sensor, interstitial glucose (IG) concentration, which reflects the blood glucose (BG) concentration.[10,20,21] CGMSs provide sensor glucose levels in real time and allow detection of hyperglycemic and hypoglycemic episodes which might otherwise be undetected.[22] Commercially available sensors have a functional life of up to 14 days and are well tolerated by DD.[15] A novel CGMS equipped with a long-term sensor (Eversense XL; Senseonics, Inc., Germantown, Maryland) has recently been licensed for use in the European Union (CE marking in 2017).[23] This system consists of a fully implanted sensor, a wearable transmitter, and a mobile application (Figure 1).[23]

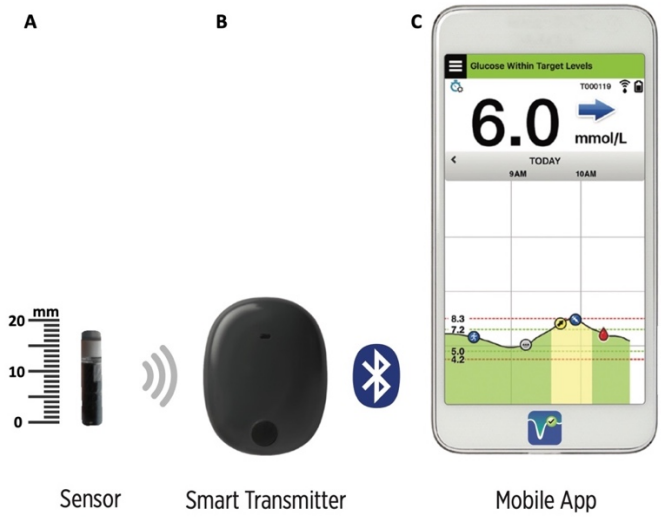

Figure 1. Eversense XL consists of the following: (**A**) the sensor (18.3 mm in length and 3.5 mm in diameter) which is implanted in the subcutaneous tissue; (**B**) the smart transmitter; (**C**) the mobile application which displays glucose information on a handheld device.

The main advantages of Eversense XL are the extended life of up to 180 days, the reduced need for sensor replacement, and the flexibility of being able to remove the external transmitter.[23-25] However, unlike the transcutaneous CGMSs, the long-term sensor has to be implanted and removed from the skin by means of a minimally invasive surgical procedure performed by a health care professional.[23] Recent investigations have shown that Eversense is safe and accurate for use in humans with diabetes, the overall mean absolute relative difference (MARD) ranges from 8.5 to 9.4% and the 20/20% agreement rate is 93 and 99% of values in zones A and B on the Clarke Error Grid, respectively.[25–29] To date, no studies have evaluated the use of the long-term sensors in DD. The present case series describes, for the first time, the clinical use of Eversense XL in three DD, and the correlation between IG measured by Eversense XL, IG measured by a flash glucose monitoring system (FGMS; FreeStyle Libre, Abbott, UK) and BG measured by a portable-blood glucose meter (PBGM; Alphatrak2, Zoetis/Abbott, UK) previously validated for use in DD.[15,30,31]

CLINICAL CASES

2.1. Case Descriptions

2.1.1. Dog 1

A 14-year, 2-month-old, 5.3 kg male neutered Yorkshire Terrier was presented to the Veterinary Teaching Hospital of the University of Bologna (UVTH) for a routine re-evaluation of diabetes. The dog had been diagnosed with DM 5 years earlier and, at the time of the presentation, was on a moderate-carbohydrate, moderate-fiber prescription diet (Diabetic Royal Canin, Royal Canin SAS, Milano, Italy) and 4 U of porcine lente insulin (Caninsulin, MSD, Boxmeer, The Netherlands), twice daily. The owner described the absence of symptoms related to DM (e.g., polyuria, polydipsia, polyphagia, weight loss), and was monitoring the BG of the dog by means of blood glucose curves (BGCs) at home, using the PBGM. In addition, the owner often applied the FGMS at least once a month to monitor the IG continuously. On physical examination, the dog had a normal body condition score (BCS, 5/9) and a mature bilateral cataract. The CBC and the biochemistry profile were unremarkable. Urinalysis showed a urinary specific gravity (USG) of 1.030 and glycosuria.

2.1.2. Dog 2

A 12-year, 6-month-old, 23.9 kg male neutered English Setter was presented to the UVTH for a re-evaluation of diabetic control. The dog had been diagnosed with DM 1 year earlier and, at the time of the presentation, was on a moderate-carbohydrate, high-fiber homemade diet and 14 U of porcine lente insulin, twice daily. The DM-related signs were not reported. The owner was monitoring the IG of the dog using the FGMS. On physical examination, the dog had a normal BCS (4/9), and no abnormalities were detected. The CBC was unremarkable and the biochemistry profile showed a marked increase in serum cholesterol (594 mg/dL, reference range [RR] 123–345) and triglycerides (672 mg/dL, RR 30–120). Urinalysis showed a USG of 1.045 and the absence of glycosuria.

2.1.3. Dog 3

A 12-year, 7-month-old, 15.3 kg female spayed mixed breed dog was presented to the UVTH for a routine re-evaluation of DM. The dog had been diagnosed with DM 2 years earlier and, at the time of the presentation, was on a moderate-carbohydrate, moderate-fiber prescription diet (Diabetic Royal Canin) and 6 U of neutral protamine Hagedorn (NPH) insulin (Humulin I, Eli Lilly, Sesto Fiorentino, Italy), twice daily. In addition, the dog was receiving bezafibrate (10 mg/kg; once daily) for the treatment of hyperlipidemia. At the time of presentation, the DM-related symptoms were not reported. The owner was monitoring the IG using the FGMS. On physical examination, the dog had a normal BCS (4/9) and a mild bilateral cataract. The CBC was unremarkable and the biochemistry profile showed a mild increase in alanine aminotransferase (ALT) (106 U/l, RR 15–65) and serum cholesterol (409 mg/dL, RR 123–345). Moreover, a moderate increase of serum triglycerides (417 mg/dL, RR 30–120) was also detected. Urinalysis was not performed.

2.2. The Eversense Long-Term Implantable CGMS

The components of the Eversense XL are shown in Figure 1. The sensor is encased in biocompatible material and utilizes a unique fluorescent, glucose-indicating polymer. A light-emitting diode embedded in the sensor excites the polymer, and the polymer then rapidly signals changes in glucose concentration via a change in light output. The measurement is then relayed to the smart transmitter. The sensor has a silicon collar containing dexamethasone, which is slowly released to reduce the inflammation which could degrade sensor functioning. The transmitter is a reusable device worn externally over the inserted sensor which powers the sensor and sends glucose information to the mobile application via bluetooth low-energy technology every five minutes. It is held in place with a mild silicone-based adhesive and is rechargeable via a micro-USB cable. The transmitter and sensor use an inductive link to communicate across the skin (near-field communication). The mobile application needs to be run on a compatible handheld device to receive and display the sensor glucose data from the smart transmitter. The data are stored, for up to a year, on a cloud-based platform

and are analyzed by dedicated software (Data Management System, DMS) which is easily accessed by patients and health care providers and generates summary glucose reports (ambulatory glucose profile and other customized reports) [23–25]. The detection limits of the sensor are between 40 and 400 mg/dL; when the IG concentration is <40 mg/dL and >400 mg/dL, the mobile application shows “LO” and “HI,” respectively.

2.3. Sensor Insertion and Follow-Up Assessments

Due to the high motivation of the owners and the good attitude of the three dogs to wearing the FGMS, the subcutaneous insertion of Eversense XL was proposed; written informed consent was obtained. The sensor insertion was performed as described in Figure 2.

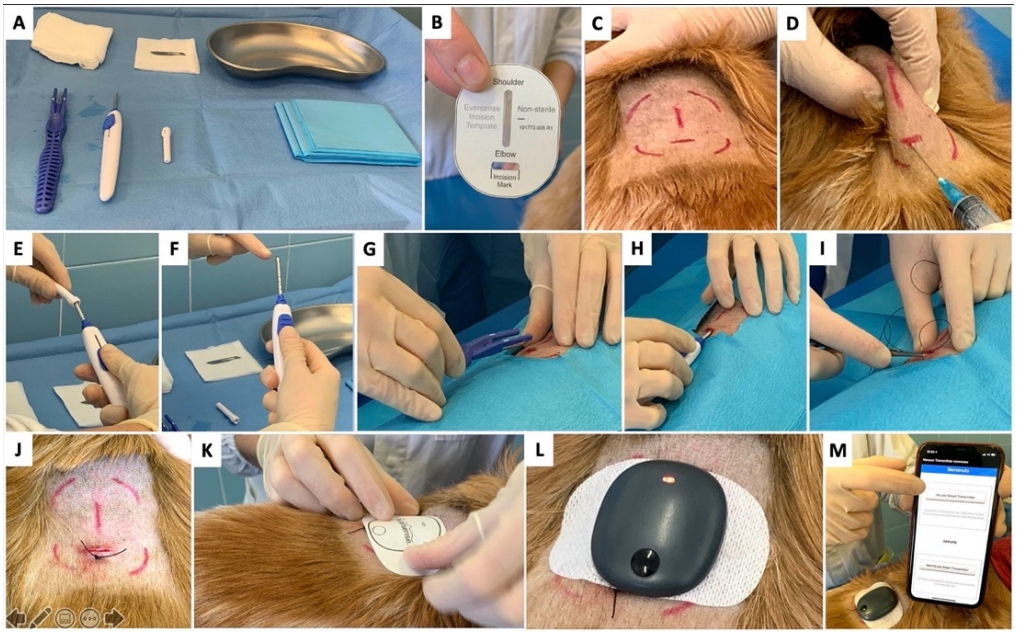

Figure 2. Eversense XL insertion in a diabetic dog. (**A**) Eversense XL sensor pack, blunt dissector and insertion tool with the necessary equipment: sterile cloth, gauze pads (3 with alcoholic chlorhexidine), and scalpel blade; (**B**) the incision template which is used to guide and mark the incision area on the skin surface by aligning the marking template to the marked outer edges of the smart transmitter; (**C**) the skin is trichotomized, marked using the incision template, and cleaned with chlorhexidine (**D**) local anesthesia (lidocaine) is injected along the planned incision site; (**E**) the sensor holder is slid into the insertion tool; (**F**) the sensor is secured inside the insertion tool; (**G**) Once the insertion area is sufficiently anesthetized, a small incision of 5–8 mm is made and, a subcutaneous pocket is created to accommodate the sensor using the blunt dissector. (**H**) the sensor is placed in the subcutaneous pocket making use of the insertion tool; (**I**) the skin is closed with non-absorbable sutures; (**J**) the sensor is now in place and ready for connection with the smart transmitter; (**K**) an adhesive patch, which attaches to the skin and to the back of the smart transmitter, is applied over the insertion site; (**L**) the smart

In dogs 1 and 2, the sensor was inserted in the lateral aspect of the thorax (Figure 3A,B). In dog 3, the sensor was implanted in the dorsal aspect of the thorax (Figure 3C).

Figure 3. (**A**) Dog 1 wearing Eversense XL (**left**) and FreeStyle Libre (**right**); (**B**) Dog 2 wearing Eversense XL; (**C**) Dog 3 wearing Eversense XL (**right**) and FreeStyle Libre (**left**).

In dog 1, due to the dog's poor compliance, the procedure was performed under brief general anesthesia while, in dogs 2 and 3, the procedure was performed under local anesthesia. In the latter case, no signs of discomfort or pain were noted. The device initialization phase consisted of 4 glucose calibration tests carried out 2 to 12 h apart. The device was subsequently calibrated every 12 h by the owner using BG or IG, measured by the PBGM and the FGMS, respectively.

No serious adverse events (AEs) were reported in any of the dogs. In dog 1, a mild erythema in the site of application of the FGMS was noted. In dogs 1 and 3, mild and moderate dislocation of the sensor from the implantation site were noted, respectively. In dog 2, excessive dislocation (i.e., movement of the sensor away from the implantation site) of the sensor affecting the connection with the transmitter was noted. The glucose data were monthly remotely assessed using the cloud-based Eversense DMS and the treatment was changed accordingly. The follow-up visits were scheduled at 90 and 180 days after the sensor implantation. At the time of the last follow-up visit, the owners of dogs 1 and 3 were very satisfied (N 5 on the 5-point satisfaction scale). Dog 1 had no diabetes-related clinical signs and was still receiving 4 U of porcine lente insulin. Dog 3 also did not show clinical signs, and the dose of NPH insulin was 7 U. In dogs 1 and 3, the sensor remained functional throughout the entire expected wear time and, at the end of its lifespan, it was not removed from the subcutaneous tissue. In dog 2, after 20 days, the owner reported trouble with daily calibrations due to sensor dislocation. At the 90-day follow-up visit, the owner was no longer using the Eversense CGM; however, the device was not removed from the subcutaneous tissue.

2.4. Glucose Data Analysis

Glucose data, automatically calculated by the Eversense DMS, are shown in Table 1 and Figure 4. Eversense DMS data was not available for dog 2, due to the intermittency of recording and short wearing time of the sensor.

Glucose (mg/dL)	DOG 1	DOG 3
Mean glucose (± SD [1])	119 ± 49.8	249 ± 87.4
Lowest sensor glucose	40	44
Highest sensor glucose	350	399
Total number of glucose values	22.022	9.151
% within glucose target (70–250)	86.1	47.4
% below glucose target (<70)	11.9	0.2
% above glucose target (>250)	2.1	52.3
% below low-glucose alert (<60)	8.8	-
% above high-glucose alert (>350)	-	14.1

[1] SD, standard deviation.

Table 1. Glucose data in two dogs with diabetes mellitus at the end of the sensor

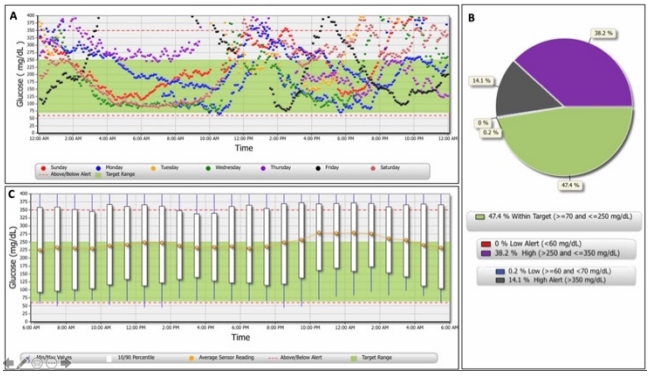

Figure 4. Glucose reports generated by the Eversense Data Management System: (**A**) Glucose trend, divided by days, over a 7-day period. Individual days can be added or removed according to the preferences of the health care providers; (**B**) Glucose pie chart showing the percentage of glucose readings within set ranges over a 180-day period; (**C**) Glucose variability reports over a 180-day period. The legend is represented in the lower part of each report.

Due to the lack of data regarding the accuracy of Eversense XL in DD, the glucose concentrations were measured in all three dogs at least once a month by means of BGCs or using the FGMS. In order to compare the IG detected by Eversense XL with both the IG detected using the FGMS and the BG detected using the PBGM (Alphatrak2), paired glucose values were collected. The BG was measured from the inner pinna with the PBGM, and simultaneously (within 1 min) the IG detected by Eversense XL was recorded from the mobile application. The simultaneous IG levels provided by the mobile applications of Eversense XL and the FGMS was recorded. The owners were allowed to obtain paired

measurements during the entire wearing period of Eversense XL. The measurements obtained for the calibration tests and all glucose concentrations above or below the detection limit of the sensor (<40 and >400 mg/dL, respectively) were excluded from the analysis.

The normality of the glucose values was assessed using the Shapiro–Wilk test, and nonparametric tests were used accordingly. The correlation between the glucose concentrations measured by Eversense XL and those measured by the FGMS and the PBGM was evaluated using Spearman's rank correlation. The differences between the BG measured by the PBGM and the IG measured by Eversense XL were plotted against the reference values in Bland–Altman plots. Analytical accuracy was determined by calculating the mean absolute relative difference (MARD), median absolute relative difference (mARD), mean absolute difference (MAD), and mean relative difference (MRD).[32] All these are measures of the average difference between Eversense XL and reference results (PBGM). MARD and mARD measure the size but not the direction (higher or lower) of the differences compared with the reference (absolute) as a percentage of the reference value (relative). MAD is similar, but just reports the size of the difference (it is not reported as a percentage), and is commonly used to assess accuracy at low BG values (<100 mg/dL). MRD measures the size and direction of the difference compared with the reference as a percentage of the reference value. Second, analytical accuracy was estimated based on ISO 15197:2013 criteria, which state that at least 95% of results must be within ±15 mg/dL of the BG concentration for BG concentration <100 mg/dL and within ±15% of the BG concentration for BG concentration ≥100 mg/dL. In addition, the precision absolute relative difference (PARD) was calculated. Instead of sensor-to-BG differences, the sensor-to-sensor differences (Eversense XL vs. FGMS) were calculated as the difference between sensor readings divided by the average of the sensors' readings.[33]

Parkes Consensus Error Grid analysis (EGA) for type 1 DM was performed to assess clinical risks for each measurement, and the values of glucose concentrations measured by the reference method (PBGM) were assigned to the x-axis versus glucose concentrations measured by Eversense XL on the y-axis in eight concentric zones with no discontinuities (A through E) defined by different lines.[34]

A total of 264 paired glucose results were obtained, of which 66% (175/264) were obtained with the FGMS and 34% (89/264) with the PBGM. A strong positive correlation was found between the IG measured by Eversense XL and the FGMS (r_s = 0.85; 95% confidence interval [CI], 0.80–0.89; $p < 0.0001$), as well as between the IG measured by Eversense XL and the BG obtained using the PBGM (r_s = 0.81; 95% CI, 0.72–0.87; $p < 0.0001$). The mean ± standard deviation (SD) differences between the BG and the IG were −31.5 ± 54.5 mg/dL (95% limits of agreement, −138.3–75.2; Figure 5). The results of Eversense's analytical accuracy in the low (BG < 100 mg/dL) and high glucose range (BG ≥ 100 mg/dL) are shown in Table 2. The PARD was 26.3%.

Low Glucose Range (BG < 100 mg/dL)	
Number of glucose values	17
MAD (mg/dL)	17.4
Percent of values within ±15 mg/dL of the BG value	52.9% (9/17)
High Glucose Range (BG > 100 mg/dL)	
Number of glucose values	72
MARD (%)	24.5
mARD (%)	20.5
MRD (%)	−18.7
Percent of values within ±15% of the BG value	41.7% (30/72)

Abbreviations: BG, blood glucose; MAD, mean absolute difference; MARD, mean absolute relative difference; mARD, median absolute relative difference; MRD, mean relative difference.

Table 2. Eversense's analytical accuracy in the low (BG < 100 mg/dL) and high glucose range (BG ≥ 100 mg/dL).

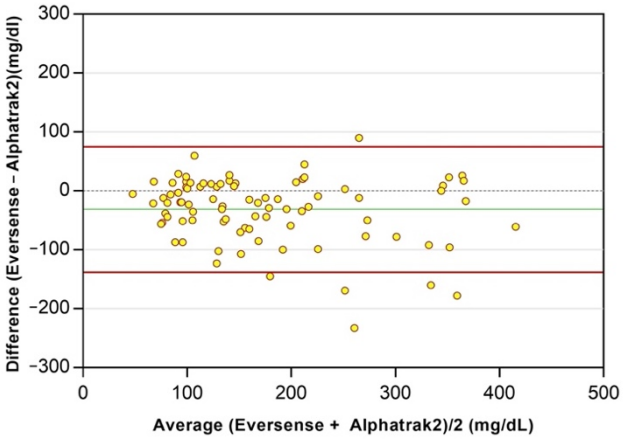

Figure 5. Bland–Altman plots represent the differences between the glucose concentrations obtained using Eversense XL versus those obtained using the PBGM (Alphatrak2) in all dogs. The PBGM glucose values plotted against absolute errors for each corresponding value are on the x-axis. The black dotted line represents a mean difference of 0 between the glucose concentrations being compared. The green line represents the mean difference between the glucose concentrations being compared, and the red lines represent the 95% limits of agreement.

Evaluation of data using the Parkes consensus EGA showed that 95.5% of the Eversense XL results fell in zones A and B (Figure 6).

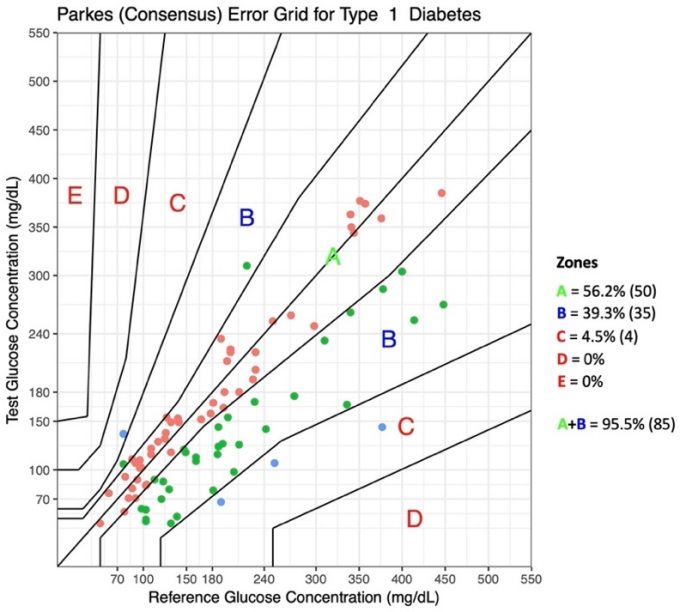

Figure 6. Parkes consensus error grid analysis (EGA) representation with the percentage of values within different zones. The reference glucose values (blood glucose obtained by a portable glucometer) on the x-axis are plotted against the interstitial glucose measurements obtained by the Eversense XL on the y-axis. The different zones designate the magnitude of risk: no effect on clinical action (Zone A); altered clinical action, little or no effect on the clinical outcome (Zone B), altered clinical action, likely to affect the clinical outcome (Zone C); altered clinical action, could have a significant medical risk (Zone D); and altered clinical action, could have dangerous consequences (Zone E). ISO 15197:2013 requires that 99% of the values fall within Zones A + B for a device to be considered accurate.

DISCUSSION

The clinical use of Eversense XL resulted in continuous IG monitoring over a 180-day period and high user satisfaction in 2/3 DD. The results showed a good correlation between the glucose concentrations measured by Eversense XL and those obtained using a PBGM and a FGMS, previously validated for use in DD.[15,30,31] Eversense XL is an innovative system for monitoring IG in patients with diabetes.[23] This device overcomes some of the limitations of transcutaneous CGMSs, such as trouble inserting the sensor, insertion pain, the burden of frequent sensor replacement, discomfort from wearing the sensor, dissatisfaction with wearing diabetes devices, sensor dislodgement and skin irritation.[24,35,36] In the present study, the device was well tolerated by all the dogs, and no serious AEs (i.e., discomfort, local and systemic signs of inflammation) were recorded. It is worth mentioning that mild erythema at the site of the application of the FGMS was noted in one of the dogs. Dermatologic complications associated with the use of FreeStyle Libre have been reported in up to 80% and 18% of DD and cats, respectively.[15,37,38] Allergic contact dermatitis, likely caused by the sensor's built-in adhesive, is a known problem in human medicine and, in a retrospective study, it was observed in 3.8 % of 1036 diabetic patients using FreeStyle Libre.[39] In a recent study, the incidence of device-related AEs was low in 3023 patients using Eversense XL [29]. In that study, the most frequently reported AEs were sensor location site infection (0.96%), inability to remove the sensor at the first attempt (0.76%) and adhesive patch location site irritation (0.66%).[29]

In the current study, sensor dislocation and trouble with daily calibration were the only device-related AEs reported by the owners. However, since the sensor was not removed at the end of the wearing period, it was not possible to assess whether the inability to remove the sensor could represent a device-related drawback also in DD. The dislocation of the sensor from the implantation site has not been described in humans.[29] Potential reasons for this discrepancy were the different sensor implantation sites (i.e., the thorax in dogs as compared with the upper arm in humans) and the anatomic differences between human and dog skin.[23,40] Of note is that the external surface of the implanted sensor has a silicone collar which slowly releases the dexamethasone acetate into the adjacent subcutaneous tissue to suppress inflammation and the foreign body immune response.[41] This safety mechanism does not allow the formation of a surrounding fibrous capsule which could potentially block the sensor at the implantation site. Sensor movement was less marked in the only small-breed dog included (Dog 1), suggesting individual variability possibly related to dog breed and size. The dislocation of the sensor makes it difficult to carry out daily calibration tests since connection between the sensor and the transmitter is possible only when the transmitter is positioned directly over the sensor. Furthermore, when daily calibration tests are not completed within a 24-h period, the system re-enters the initialization phase.[42] Based on the above, a constant commitment from the owner is required for management of the device.

In the present study, although only dogs accustomed to wearing the FGMS and motivated owners were selected, in one of the three dogs, the Eversense XL was used only for a limited period. This highlights the importance of careful dog selection when this device is used. In addition, the high cost and limited availability of the device are additional factors which should be taken into consideration. The application of the sensor was minimally invasive, quick and simple owing to the insertion tools provided by the manufacturer. In two dogs, the sensor was inserted under local anesthesia whereas, in one case, due to the dog's poor compliance, the procedure was performed under general anesthesia. When the procedure was carried out under local anesthesia, no signs of discomfort or pain were noted. In contrast with the FreeStyle Libre, which is often applied on the neck to allow the sensor to be secured with a protective bandage, in the present cases, the thorax was chosen as a positioning site since it allowed for easy implantation of the sensor. Moreover, since the transmitter can be easily removed from the skin, it is not essential to apply a protective bandage.

We found a strong positive correlation between the glucose concentration measured by Eversense XL and that measured by the FGMS and the PBGM. However, the correlation coefficient found in this study was slightly lower compared with those of previous studies validating the FGMS in outpatient DD (r = 0.94 and 0.93).[15,31] Furthermore, the mean difference between BG and IG was greater than previously reported (Eversense XL, −31.5 ± 54.5 vs. the FGMS, 2.3 ± 46.8 and 17.2 ± 39.0).[15,31] Possible explanations for this discrepancy are differences in the gold standard methodology for BG measurement (PBGM vs. automated biochemistry analyzer) and in the number of dogs included. Our study is the first to report some preliminary results regarding the clinical and analytical accuracy of the Eversense XL in DD. The device does not fulfill the ISO 2013 accuracy requirements and MARD was higher than that previously reported in humans with diabetes (24.5% vs. 8.5 to 9.4%).[25–29] Although an accuracy analysis was conducted, the validation of Eversense XL was beyond the scope of this study given the small sample size and the absence of a standardized protocol for measuring paired glucose values. Additional studies, with a larger cohort of dogs, are needed to determine the clinical and analytical accuracy of this device in DD.

CONCLUSIONS

In conclusion, this novel long-term implantable CGMS appeared to be well tolerated and strongly correlated with two commercially available devices previously validated for use in DD. In general, the Eversense XL might be considered a future alternative for home glucose monitoring and could positively impact the adherence and long-term use of CGMSs in DD. However, the use of this device in veterinary medicine could have some limitations, such as excessive movement of the sensor, the need for daily calibrations, high costs, and limited availability. Further investigations are needed to determine the accuracy of the Eversense XL in DD.

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Chapter 4 – Canine Hypoadrenocorticism

4.1 | Prevalence of eunatremic, eukalemic hypoadrenocorticism in dogs with signs of chronic gastrointestinal disease and risk of misdiagnosis after previous glucocorticoid administration

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ABSTRACT

Background

Dogs with eunatraemic, eukalaemic hypoadrenocorticism (EEH) typically show signs of chronic gastrointestinal disease (CGD). Previous glucocorticoid administration (PGA) can give false-positive results on the ACTH stimulation test (ACTHst).

Hypothesis/Objectives

To determine the prevalence of EEH in dogs with signs of CGD, and to identify clinical and clinicopathological features for EEH and PGA.

Animals

One hundred twelve dogs with CGD (101 non-PGA and 11 PGA), 20 dogs with EEH.

Methods

Multicenter prospective cohort study. Basal serum cortisol (BSC) concentration was measured in dogs with signs of CGD. When BSC was <2 μg/dL and in PGA dogs, ACTHst plus measurement of endogenous ACTH (eACTH) were performed. Records of dogs with EEH from 2009 to 2021 were reviewed.

Results

The BSC concentration was <2 μg/dL in 48/101 (47.5%) non-PGA and in 9/11 (82%) PGA dogs. EEH was diagnosed in 1/112 dog (prevalence 0.9%;95%CI, 0.1%-4.8%); the ACTHst provided false-positive results in 2/11 PGA dogs. PGA dogs showed lower C-reactive protein-to-haptoglobin ratio (median 0.01, range 0.003-0.08;P=.01), and higher haptoglobin (140, 26-285 mg/dL;P=.002) than non-PGA dogs (0.04, 0.007-1.5; 38.5, 1-246 mg/dL; respectively). eACTH was higher (P=.03) in EEH (396, 5->1250 pg/mL) than in non-PGA dogs (13.5, 7.3-46.6 pg/mL). Cortisol-to-ACTH ratio was lower (P<.0001 and P=.01,respectively) in EEH (0.002, 0.0002-0.2) than in non-PGA (0.1, 0.02-0.2) and PGA dogs (0.1, 0.02-0.2).

Conclusions and clinical importance

The prevalence of EEH in dogs with signs of CGD was lower than previously reported. The clinical and clinicopathological features herein identified could increase the index of suspicion for EEH or PGA in dogs with an unclear history of glucocorticoid administration.

INTRODUCTION

Hypoadrenocorticism (HA) is a rare endocrinopathy in dogs.1 Primary HA refers to bilateral adrenal gland destruction and accounts for more than 95% of cases.[2] Secondary HA, a much rarer condition, is due to reduced ACTH secretion from the pituitary gland.[2] In the majority of cases of primary HA, both glucocorticoid and mineralocorticoid secretions are impaired, resulting in hypocortisolemia and electrolyte abnormalities; nevertheless, up to 30% of dogs with primary HA have normal electrolyte concentrations at diagnosis.[3-5] This form of the disease is therefore defined as eunatraemic, eukalaemic hypoadrenocorticism (EEH), also defined as "atypical" hypoadrenocorticism.[6] Dogs with EEH are characterized by a condition of permanent hypocortisolemia usually associated with a low to undetectable aldosterone concentration, despite having normal electrolyte concentrations.[7,8] Eunatraemic, eukalaemic hypoadrenocorticism might go undetected for a long period due to vague clinical signs and the absence of typical biochemical abnormalities. Consequently, EEH might be mistaken for other diseases, such as chronic gastrointestinal disease (CGD). Diagnosing HA depends on adrenal gland function testing, such as the ACTH-stimulation test (ACTHst). However, there have recently been problems with this test, including its high cost and the intermittent availability of exogenous ACTH in some countries.[9,10] As a result, basal serum cortisol (BSC) concentration, using a cut-off value of ≥2 µg/dL (>55 nmol/L), is commonly used as a screening test to rule out HA. BSC concentration <2 µg/dL (<55 nmol/L) has excellent sensitivity for HA (99.4%-100%).[11-13] However, due to the low specificity of the test (20%-78.2%), up to 33% of dogs with CGD, but without HA, have a BSC <2 µg/dL (<55 nmol/L).11-16 For this reason, urine cortisol-to-creatinine ratio (UCCR) and cortisol-to-ACTH ratio (CAR) have been proposed as alternative screening tests for HA in dogs.[14,17-19]

Even though the ACTHst remains the gold standard for HA diagnosis, previous glucocorticoid administration (PGA), commonly used in dogs with signs of CGD, can give false positive results. In previous studies, PGA was excluded based only on the history of the dog which likely led to an overdiagnosis of EEH.[3-5,15] Demonstrating a high endogenous ACTH concentration (eACTH) could be an objective method for differentiating EEH from false positive results of the ACTHst due to PGA. Dogs with secondary HA have low eACTH; however, this is a rare condition with marginal clinical relevance.[2] To confirm the diagnosis of EEH, it is appropriate to consider the anamnesis and the result of ACTHst; however, at the same time, it is important to demonstrate that eACTH is elevated. This study aimed to determine the prevalence of EEH in dogs with signs of CGD and to identify the clinical and clinicopathological features which might help in differentiating dogs with "atypical" hypoadrenocorticism from those with CGD, and to recognize PGA in dogs with CGD.

MATERIALS AND METHODS

Study design

A multicenter prospective cohort study involving client-owned dogs with chronic (>3 weeks) signs routinely seen in dogs with HA, such as vomiting, diarrhea, decreased appetite, weakness or lethargy, from 2 different veterinary hospitals (Veterinary Teaching Hospital of the University of Bologna, Veterinary Teaching Hospital of the University of Lisbon) from June 2019 to December 2021 was carried out. The presence of at least one of vomiting or diarrhea was a mandatory inclusion criterion. All the dogs were enroled according to the study protocol which was approved by the Scientific Ethics Committee of the University of Bologna (no. 1255/2021) . In addition, due to the low prevalence of EEH in the dogs with signs of CGD, the medical records of all the dogs with a diagnosis of EEH admitted to the Veterinary Teaching Hospital of the University of Bologna between January 2009 and December 2021 were reviewed.

Animals

The data obtained at the time of enrolment included signalment, history (including previous administration of glucocorticoids), physical examination findings, and laboratory test results which included CBC, serum chemistry profile and urinalysis. The diagnostic workup included measurement of the BSC, and determination of the folate and cobalamin concentrations. When the BSC was <2 μg/dL and in the dogs with PGA, an ACTHst plus the measurement of eACTH were carried out. Fecal flotation and standard egg count were performed in all the dogs if recent fecal testing results were not available. The decision regarding additional diagnostics was the responsibility of the clinician managing the case.

The dogs were divided into two groups: those which had not received glucocorticoids in the previous 90 days (non-PGA) and those which received systemic or topical glucocorticoids which had been suspended for fewer than 90 days before admission (PGA). In the PGA dogs, the ACTHst and the measurement of eACTH were repeated if the post-ACTH serum cortisol concentration was <3 μg/dL (<83 nmol/L). The time elapsed for repeating the tests was dictated by the dog's clinical signs or the owner's willingness to return for the tests.

A diagnosis of EEH was made if the following criteria were met: 1) post-ACTH serum cortisol concentration <2.0 μg/dL (<55.0 nmol/l); 2) high (>58 pg/ml) or undetectable (<5 pg/ml) plasma eACTH concentrations, and 3) the absence of electrolyte abnormalities. Dogs with undetectable eACTH were excluded from the EEH group if a glucocorticoid medication had been administered within 90 days before testing.

Endocrine testing and analytical procedures

For the ACTHst, blood samples were taken before and 60 minutes after the IV injection of 5 μg/kg synthetic ACTH (Synacthen, Alfasigma S.P.A., Bologna, Italy). Blood samples for the determination of eACTH concentrations were collected before the injection of synthetic ACTH. The BSC and eACTH concentrations were used for calculating the CAR. All the analytical procedures were carried out at the veterinary laboratory of the University of Bologna. The samples from Lisbon were stored at -80°C and shipped overnight on dry ice to the veterinary laboratory of the University of Bologna. Blood samples for the determination of the eACTH were collected into EDTA-coated plastic tubes placed on ice. The samples were immediately centrifuged at 4°C, 500g for 8 minutes, and the plasma was immediately transferred to plastic tubes, stored at 4°C and analyzed within 8 hours, or stored at -80°C and thawed immediately before analysis. Blood samples for the determination of the cortisol were collected in serum separating tubes. Coagulated blood samples were centrifuged for 10 minutes at 3000g; the serum was immediately transferred to plastic tubes, stored at 4°C and analyzed the same day, or stored at -80°C and thawed immediately before analysis. The serum cortisol and eACTH concentrations were measured using a chemiluminescent enzyme immunoassay (Immulite 2000, Siemens Healthcare) which had been validated for dogs and is widely used in laboratories throughout the world.[20,21]

Statistical Analysis

Statistical analysis was carried out using commercial statistical software packages (GraphPad Prism 7, San Diego, California). Descriptive statistics were generated to characterize the study population. Continuous variables were presented as median and range (minimum and maximum value). The categorical variables were described with frequencies, proportions or percentages. The overall prevalence of EEH and its 95% confidence interval (CI) according to Wilson were calculated. The differences between the groups (non-PGA, PGA, and EEH) regarding the categorical and numerical variables were analyzed using the Fisher's exact test and the Kruskal–Wallis test with Dunn's post-test, respectively. For dogs with cortisol values reported as "<1 μg/dL", 0.5 μg/dL was used for statistical calculations. For ACTH values reported as "<5 pg/mL" and ">1250 pg/mL," 5 pg/mL and 1250 pg/mL were used for calculations, respectively. The level of significance was set at $P < .05$.

Results

Animals

A total of 112 dogs were enrolled, including 101 non-PGA and 11 PGA dogs. One dog was diagnosed with EEH, giving a prevalence estimate of "atypical" HA in this cohort of dogs of 0.9% (95% CI, 0.1%-4.8%). Sixty-nine dogs were male, of which 41 were neutered, and 43 were female, of which 22 were spayed. The median age was 3.0 years (range, 6 months to 12.1 years) and the median body weight was 21.9 kg (range, 2.2-53.5 kg). Mixed breeds (n = 28) were most common, followed by Labrador Retrievers (n = 7), German Shepherds (n = 7), Jack Russell Terriers (n = 7), Weimaraners (n = 5), French Bulldogs (n = 4), and 54 other purebred dogs of 33 different breeds. The most common clinical signs on presentation are reported in Table 1. Polyuria and polydipsia (PU/PD) were more commonly reported in the PGA than the non-PGA dogs (P=.01).

TABLE 1 Descriptive statistics of clinical signs in dogs with signs of chronic gastrointestinal disease which had not received glucocorticoids (non-PGA), dogs with previous glucocorticoid administration (PGA), and dogs with eunatremic, eukalemic hypoadrenocorticism (EEH).

	Non-PGA	PGA	EEH
	n	n	n
Variable	%	%	%
Vomiting	69 69	9 81	14 70
Diarrhea	83 83	11 100	16 80
Weakness or lethargy	12[a] 12	4 36	12[a] 60
Decreased appetite	29 [a] 29	6 55	13[a] 65
Anorexia	13 13	1 9	0 -
Weight loss	30 30	3 27	2 10
Hematochezia	26 26	1 9	2 10
Melena	5 5	0 -	0 -
Polyuria/polydipsia	3[a] 3	3[a,b] 27	0[b] -

Note: Data are presented as frequencies and percentages. Significant differences ($P < .05$) between groups are shown with the same superscript symbols (a-b).

In the PGA dogs, prednisolone was the most commonly used medication in 8/11, followed by prednisone and topical triamcinolone (otic and cutaneous routes), and betamethasone (ophthalmic route), and hydrocortisone aceponate (cutaneous route) in one dog each. The median dose was 0.5 mg/kg (range, 0.14-0.9). The median time of glucocorticoid treatment and discontinuation was 60 (range, 2-300) and 25 (range, 6-63) days, respectively.

Dogs with EEH

Eunatraemic, eukalaemic hypoadrenocorticism was the final diagnosis in one dog, a 7-year-old female spayed Miniature Pinscher with a 6-month history of weight loss, vomiting, diarrhea, and sporadic episodes (2-3 times a month) of hematemesis and hematochezia. In addition, data from 19 dogs with EEH were retrospectively collected. Ten dogs were male, of which 3 were neutered, and 9 were female, all of which were spayed. Eleven different breeds were counted. The most represented breeds were mixed breed (n = 6) and Jack Russell Terrier (3), followed by one each of Golden Retriever, Pekingese, Samoyed, Siberian Husky, Boxer, Labrador Retriever, Ibizan Hound, German Shepherd, Pomeranian, and Border Collie. The median age of the dogs with EEH was 5.4 years (range, 1.3-11.7 years) and the median body weight was 19 kg (range, 3.7-36.2 kg). The clinical signs are reported in Table 1. Decreased appetite

(P=.003) and weakness or lethargy (P<.0001) were more commonly reported in EEH dogs when compared with non-PGA dogs while PU/PD were more common in PGA than in EEH dogs (P=.04).

Endogenous ACTH concentration was available in 16/19 (84%) dogs. Based on eACTH concentration, primary and secondary EEH was diagnosed in 11/16 (69%) and 5/16 (31%) dogs, respectively. Endogenous ACTH measurement was not available in the remaining 3 dogs. All the dogs diagnosed with EEH were treated with glucocorticoids and did not receive mineralocorticoid replacement. One or more concurrent diseases were documented in 12/19 (63%) dogs, including seven (37%) with primary inflammatory enteropathy (6/7 food-responsive enteropathy and 1/7 immunosuppressant-responsive enteropathy); two (10%) with hypothyroidism; and one each with diabetes mellitus, immune-mediated thrombocytopenia, lymphoma, or cutaneous mast cell tumors. Follow-up information was available in 12/19 (63%) dogs with a median follow-up time of 284 days (range, 31-2201). Of these, none of the dogs developed electrolytes abnormalities after diagnosis.

Laboratory findings and adrenal testing

The laboratory variables of the non-PGA, PGA, and EEH dogs are shown in Table 2.

TABLE 2 Results of clinicopathological variables in dogs with signs of chronic gastrointestinal disease which had not received glucocorticoids (non-PGA), dogs with previous glucocorticoid administration (PGA), and dogs with eunatremic, eukalemic hypoadrenocorticism (EEH).

	Non-PGA		PGA		EEH		
Variable (unit)	Result (n)	Range	Result (n)	Range	Result (n)	Range	*P* value
Hematocrit (%)	50.5 (98)[a]	27.4-62	46.8 (11)	42.2-56.2	43.5 (20)[a]	34.4-55.8	**.0006**
MCV (fL)	69.1 (98)	59.7-80	70.2 (11)	65.2-78.3	68.9 (20)	62.8-76.9	.51
MCHC (g%)	34.1 (98)	31.9-37.2	34.5 (11)	32.1-36	33.6 (20)	30.8-36.4	.08
RDW (%)	12.1 (96)[a]	10.3-20.8	12.4 (11)	11.3-12.8	13.4 (20)[a]	10.6-17.7	**.01**
WBC ($\times 10^3/\mu L$)	9.7 (98)	3.3-45.2	9.9 (11)	5.8-14.5	8.9 (20)	5.1-34.6	.79
Neutrophils ($\times 10^3/\mu L$)	5.8 (98)	1.5-29.4	6.5 (11)	3.7-9.5	5.1 (20)	3.2-29.1	.63
Lymphocytes ($\times 10^3/\mu L$)	2.2 (98)	0.6-6.7	1.8 (11)	0.5-3.4	2.4 (20)	1-4.6	.10
Monocytes ($\times 10^3/\mu L$)	0.48 (98)	0.08-2.6	0.32 (11)	0.23-1.1	0.44 (20)	0.15-1.5	.23
Eosinophils ($\times 10^3/\mu L$)	0.43 (97)	0-2.3	0.18 (11)	0.07-1.7	0.35 (20)	0.06-1.9	.58
Platelets ($\times 10^3/\mu L$)	252 (98)	51-600	269 (11)	142-436	274 (20)	4-521	.39
Glucose (mg/dL)	95 (97)	62-153	95 (11)	81-114	91 (19)	50-115	.63
Fructosamine (µmol/L)	244 (74)	128-396	226 (9)	171-479	225 (13)	203-347	.79
ALT (U/L)	46 (98)	23-518	50 (11)	22-858	60.5 (20)	12-315	.22
AST (U/L)	38 (89)	12-89	32 (11)	18-66	40 (19)	13-146	.43
ALP (U/L)	39.5 (96)	11-368	51 (11)	16-309	56 (20)	12-300	.34
GGT (U/L)	3.1 (87)	0-9.4	2.3 (10)	0.3-18.8	3.2 (19)	0.8-18.8	.70
Total bilirubin (mg/dL)	0.21 (83)	0.06-0.31	0.18 (11)	0.09-0.35	0.2 (20)	0.07-03	.79
Total proteins (g/dL)	6.3 (96)	4.3-7.5	6.3 (11)	5.3-7.2	6.1 (20)	5-6.9	.05
Albumin (g/dL)	3.2 (98)[a]	1.5-3.9	3.3 (11)[b]	2.5-3.9	2.9 (20)[a,b]	1.6-3.6	**.001**
Albumin/globulin ratio	1.07 (96)[a]	0.6-3.3	1.1 (11)	0.7-1.2	0.9 (20)[a]	0.5-1.5	**.007**
Cholesterol (mg/dL)	186.5 (88)	75-403	213 (11)	134-821	174 (20)	54-381	.2
Triglycerides (mg/dL)	45.5 (76)	22-161	50 (10)	27-260	67 (15)	23-144	.69
Urea (mg/dL)	36 (96)	17-125	38 (11)	29-89	39 (20)	19-131	.25
Creatinine (mg/dL)	1.05 (98)	0.6-1.7	1 (11)	0.5-1.3	0.9 (20)	0.6-1.6	.83
CRP (mg/dL)	1.1 (77)	0.7-26.3	1.1 (9)	0.8-6.1	1.5 (12)	0.6-10.2	.4
Haptoglobin (mg/dL)	38.5 (72)[a]	1-246	140 (9)[a]	26-285	69 (10)	12-212	**.002**
CRP/Haptoglobin ratio	0.04 (72)[a]	0.007-1.5	0.01 (9)[a]	0.003-0.08	0.05 (10)	0.004-2.8	**.01**
Calcium (mg/dL)	10.2 (84)	8.2-11.3	10.3 (11)	9.6-11.4	9.9 (20)	9.3-10.6	.05
Phosphate (mg/dL)	4 (84)	1.6-8.5	4 (11)	3.4-5.9	4.1 (20)	2.7-6.2	.43
Sodium (mEq/L)	148 (98)	130-159	148	141-157	148 (20)	140-153	.39
Potassium (mEq/L)	4.4 (98)	3.7-5.3	4.1 (11)[a]	3.6-5.1	4.8 (20)[a]	3.6-5.3	**.03**
Chloride (mEq/L)	113.4 (91)	90.8-124	111 (11)	102-122	113.7 (20)	103.7-120	.14
Cobalamin (mEq/L)	436.5 (88)	150-1000	371.5 (6)	219-584	509 (8)	216-1118	.58
Folate (µg/dL)	6.3 (88)	1-24	7.2 (6)	6.5-15.4	9.7 (8)	1-18.2	.23
Urinary specific gravity	1042 (49)	1006-1080	1044 (8)	1030-1062	1042 (16)	1029-1070	.94
UPC	0.13 (26)	0.06-1.7	0.3 (6)	0.1-2.6	0.1 (15)	0.07-0.6	.13

Note: Data are presented as median and range (min-max value). The *P*-values in bold correspond to statistically significant findings ($P < .05$). Significant differences between groups are shown with the same superscript symbols (a-b).
Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate transaminase; CRP, C-reactive protein; GGT, gamma-glutamyl transferase; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RDW, red cell distribution width; UPC, urine protein-to-creatinine ratio; WBC; white blood cells.

Dogs with EEH showed significantly lower hematocrit (P=.0005), serum albumin concentration (P=.0001) and albumin-to-globulin ratio (P=.007), and higher red cell distribution width (RDW, P=.01) than non-PGA dogs. Moreover, the EEH dogs had lower serum albumin (P=.02), and higher potassium (P=.03) concentrations when compared with the PGA dogs. Haptoglobin concentration was significantly higher (P=.002) and the C-reactive protein-to-haptoglobin ratio (CHR) significantly lower (P=.01) in the PGA than in the non-PGA dogs. The adrenal test results are reported in Table 3.

TABLE 3 Results of adrenal testing in dogs with signs of chronic gastrointestinal disease which had not received glucocorticoids (non-PGA), dogs with previous glucocorticoid administration (PGA), and dogs with eunatremic, eukalemic hypoadrenocorticism (EEH).

	Non-PGA		PGA		EEH		
Variable (unit)	Result (n)	Range	Result (n)	Range	Result (n)	Range	*P* value
Basal cortisol (µg/dL)	2.1 (100)[a,b]	0.4-11.3	1.3 (9)[a]	0.5-1.7	0.6 (20)[b]	0.1-1.3	**<.0001**
Post-ACTH cortisol (µg/dL)	9.9 (47)[a]	3.2-16.8	8.3 (11)[b]	0.6-12.5	1 (20)[a,b]	0.2-1.9	**<.0001**
Endogenous ACTH (pg/mL)	13.5 (47)[a]	7.3-46.6	14.2 (11)	5-83.4	396 (17)[a]	5->1250	**.03**
Cortisol-to-ACTH ratio	0.1 (47)[a]	0.02-0.2	0.1 (9)[b]	0.02-0.2	0.002 (17)[a,b]	0.0002-0.2	**.0001**

Note: Data are presented as median and range (min-max value). The *P*-values in bold correspond to statistically significant findings ($P < .05$). Significant differences between groups are shown with the same superscript symbols (a-b).

In the non-PGA dogs, 48/101 (47.5%) had BSC <2 µg/dL (Figure 1), and only one dog was diagnosed with EEH (post-ACTH cortisol 1.51 µg/dL; eACTH >1250 pg/mL). The BSC was <2 µg/dL in all the PGA dogs in which it was measured (9/11, 82%) and was significantly lower (P=.008) when compared with the non-PGA dogs (Figure 2).

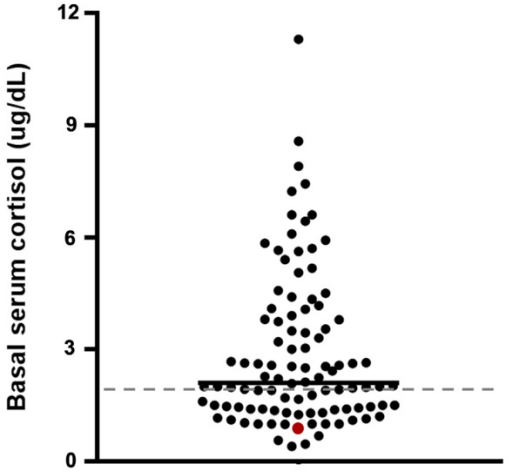

FIGURE 1 Basal serum cortisol concentration in 101 dogs with signs of chronic gastrointestinal disease which had not received glucocorticoids. The dashed gray line represents a 2 µg/dL (55 nmol/L) cut-off for excluding hypoadrenocorticism. The red dot represents the dog with the final diagnosis of eunatremic, eukalemic hypoadrenocorticism.

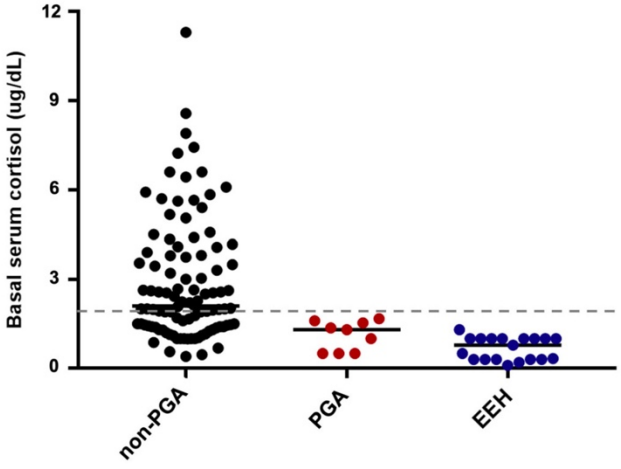

FIGURE 2 Comparison of basal serum cortisol concentration in dogs with signs of chronic gastrointestinal disease which had not received glucocorticoids (non-PGA) (n = 100), dogs with previous glucocorticoid administration (PGA) (n = 9), and dogs with eunatremic eukalemic hypoadrenocorticism (EEH) (n = 20). The dashed gray line represents a 2 µg/dL (55 nmol/L) cut-off for excluding hypoadrenocorticism. The horizontal bars represent the median values.

The ACTHst provided a false-positive result in 2/11 PGA dogs (Figure 3); the first dog had been treated with 0.3 mg/kg prednisolone for seven months for suspected EEH and, at the time of admission, the glucocorticoids had been discontinued for 6 days; a second dog had been treated for 30 days prior to the ACTHst with 0.9 mg/kg prednisolone for 4 days. In these dogs, the eACTH was low-normal (5 and 17.5 pg/mL, respectively) and the repeated ACTHst (after 14 and 33 days, respectively) was normal. The dogs with EEH showed lower BSC (P<.0001) and post-ACTH cortisol (P<.0001), and higher eACTH (P=.03; Figure 4) than the non-PGA dogs.

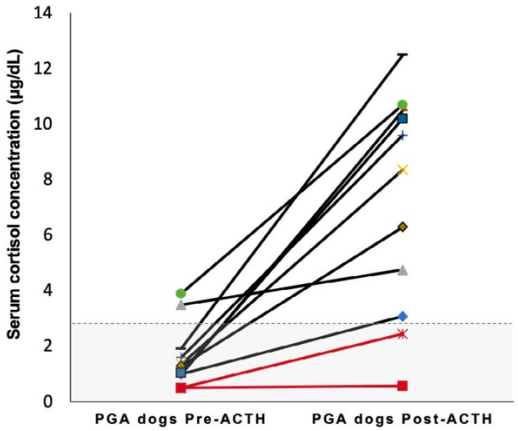

FIGURE 3 Serum cortisol concentrations before and after stimulation with synthetic ACTH in 11 dogs with previous glucocorticoid administration (PGA). The dashed gray line represents a 3 µg/dL (83 nmol/L) cut-off.

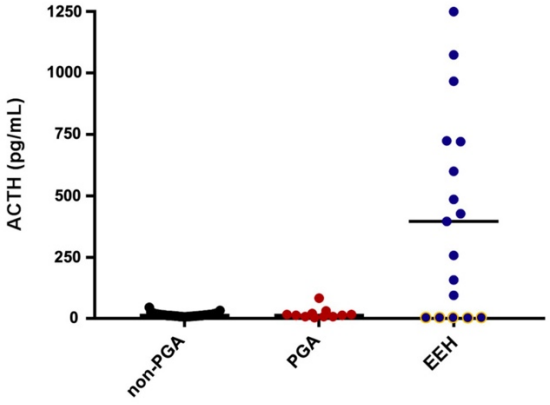

FIGURE 4 Comparison of the endogenous ACTH (eACTH) concentration in dogs with signs of chronic gastrointestinal disease which had not received glucocorticoids (non-PGA) (n = 100), dogs with previous glucocorticoid administration (PGA) (n = 11), and dogs with eunatremic eukalemic hypoadrenocorticism (EEH) (n = 20). The dots with yellow borders represent dogs with secondary hypoadrenocorticism. The horizontal bars represent the median values.

Moreover, the CAR was significantly lower (P<.0001 and P=.01, respectively) in the EEH dogs when compared with the non-PGA and the PGA dogs (Figure 5).

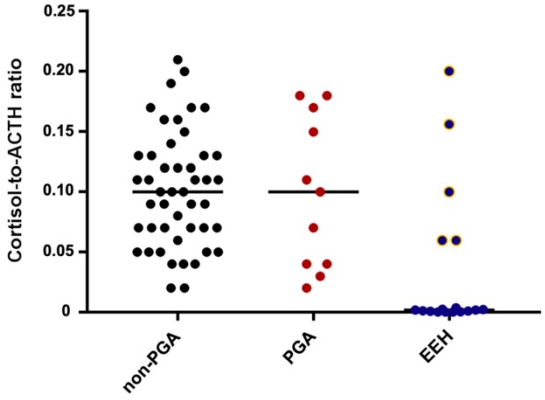

FIGURE 5 Comparison of the cortisol-to-ACTH ratio (CAR) in dogs with signs of chronic gastrointestinal diseases which had not received glucocorticoids (non-PGA) (n = 100), dogs with previous glucocorticoid administration (PGA) (n = 11), and dogs with eunatremic eukalemic hypoadrenocorticism (EEH) (n = 20). The dots with yellow borders represent dogs with secondary hypoadrenocorticism. The horizontal bars represent the median values.

DISCUSSION

In this multicenter prospective study, the prevalence of EEH in a cohort of dogs with CGD presented to two referral institutions was 0.9% (95% CI, 0.1%-4.8%). The estimated prevalence in the present study closely corresponded with the overall prevalence of HA in the general canine population (between 0.06 and 1.1%),[1,22-24] and with that recently reported in a large group of dogs with signs of CGD.16 However, the results of this study demonstrated a lower prevalence of EEH than previously described in dogs with signs of CGD presented to several referral centers in Germany and in the Netherlands.15 In the latter study, 6 of the 151 (4%) dogs with signs of CGD were diagnosed with hypoadrenocorticism and none of these dogs had abnormalities in serum electrolyte concentrations.[15] Unfortunately, as in many other studies,[5,15,16,26] eACTH was not measured which might have led to an overestimation of the true prevalence of EEH.

Moreover, it remains unknown whether those cases suffered from primary HA or if some of them might have had secondary HA. It is important to remember that the measurement of eACTH remains a cornerstone for the diagnosis of hypoadrenocorticism, especially when abnormalities in serum electrolyte concentrations are not detected.[2] Failure to evaluate this variable might result in a distorted prevalence of EEH. In addition, the differences between the two studies could have been due to the different inclusion criteria, especially with regard to previous glucocorticoid administration. In the present study, the ACTHst provided a false positive result in two dogs with PGA. It should be noted that one of the dogs had been treated with prednisolone for only four days and eACTH was found to be undetectable 30 days after glucocorticoid discontinuation. Based on these findings, the dog could have been diagnosed as having secondary hypoadrenocorticism. It is therefore important to exclude PGA in dogs with low pre- and post-ACTH cortisol concentrations. Glucocorticoids lead to suppression of the hypothalamic-pituitary-adrenal (HPA) axis and its duration varies based on preparation, dose, and individual sensitivity to steroid drugs.[25] To date, there are no published guidelines regarding the delay required for adequate HPA recovery after the administration of systemic or topical glucocorticoids. Due to the marked inter-individual variability, in the authors' opinion, it is difficult to establish the best timing for carrying out an ACTHst after glucocorticoids discontinuation. Even in this context, eACTH measurement appears to be of utmost importance as it allows the clinician to suspect iatrogenic HA.

The present study identified clinical and laboratory variables that were significantly different between the PGA dogs and the other study groups. Polyuria and polydipsia were more commonly reported in the PGA dogs when compared with the EEH and the non-PGA dogs. Glucocorticoids have the potential of causing PU/PD; this might result in an increased perception of these signs by the owner. Hence, PU/PD should raise the clinician's index of suspicion for PGA. However, PU/PD can also be caused by gastrointestinal diseases and should be interpreted carefully in a dog with signs of CGD.[27] Serum albumin and potassium concentrations were significantly different between the PGA and the EEH dogs; however, substantial overlap was observed. In the current study, serum haptoglobin concentration was significantly higher and the CHR significantly lower in the PGA than in the non-PGA dogs. Haptoglobin is a moderate acute-phase protein particularly sensitive to glucocorticoids; elevated concentrations are found both after treatment with glucocorticoids and during naturally occurring hypercortisolism.[28,29] However, haptoglobin is a positive acute-phase protein and results could be biased if a dog has a concomitant inflammatory disease such as CGD. In this context, measurement of the CHR could be useful for differentiating PGA from non-PGA dogs since exogenous glucocorticoid treatment appears to blunt the magnitude of C-reactive protein elevation in dogs with inflammatory state.[29] The finding of high serum haptoglobin concentration and low CHR in a dog with signs of CGD should alert the clinician to consider the possibility of PGA. Given the present results, these measurements should be considered to be a part of the diagnostic work-up when PGA is suspected.

In the current study, 47.5% of the non-PGA and all the PGA dogs had a BSC concentration <2 μg/dL (<55 nmoL/L). This finding was consistent with other investigations, demonstrating that up to 33% of dogs with nonadrenal illness have a BSC concentration <2 μg/dL (<55 nmoL/ L).11-16 Moreover, BSC was significantly lower in the PGA than in the non-PGA dogs. Determination of the BSC concentration has a high sensitivity (100% if <2 μg/dL) for HA, but a low specificity of only 20–78.2%.[11-14] Therefore, due to the low specificity of the test, an ACTHst should be carried out in dogs with BSC <2 μg/dL (<55 nmol/L) in order to exclude HA. In this study, an ACTHst was carried out in approximately half of the dogs with signs of CGD which are the signs most commonly screened for HA in clinical practice, with a consequent increase in diagnostic cost and time for the client. This raises the question of whether BSC concentration should be measured in all dogs with signs of CGD. For this reason, the CAR and the UCCR have been proposed as alternative screening tests for HA in dogs.[14,17,18,30] The CAR is a valuable and reliable tool for diagnosing

primary HA, the advantage of which is that only a single blood sample needed.[14,17] However, the diagnostic utility of this test can be limited in clinical practice owing to the critical sampling collection and handling needed for eACTH measurement. The present results were in agreement with previous reports,[14,17] and demonstrated that the CAR could be considered a useful diagnostic test for discriminating EEH dogs from those with signs of CGD and PGA. However, when also considering dogs with secondary HA some overlap between the groups was detected. In dogs with secondary HA, the UCCR could be more useful as compared to the CAR. Recent studies have shown the excellent diagnostic performance of the UCCR in dogs with HA, having a reported sensitivity and specificity ranging from 97.2–100% and 93.6–97.3%, respectively.[18,30] The anti-cortisol antibody used in the chemiluminescent enzyme immunoassay has recently been changed by the manufacturer, which indicates that the UCCR performance reported herein might need to be validated again with a new assay. Since the authors did not evaluate the UCCR, it is not possible to draw any conclusion regarding this test.

The present study described a large cohort of dogs with EEH in which eACTH was measured. Based on eACTH concentration, primary and secondary EEH was diagnosed in 69% and 31% of dogs, respectively. Secondary HA due to the reduced secretion of eACTH from the pituitary gland is considered a rare cause of adrenocortical failure (fewer than 5% of cases),2 and its prevalence in dogs with EEH has not been reported in any study. Dogs with EEH were more likely to have decreased appetite, weakness or lethargy when compared with non-PGA dogs. These differences reflected the fact that "atypical" cases might go undetected for a longer period since the clinical signs are non-specific and often wax and wane. However, data from EEH dogs were retrospectively collected, and these cases were selected based on their diagnosis rather than signs of CGD. This might have biased the comparison between groups with regard to clinical signs. Concurrent diseases were documented in 63% of the dogs, and the majority of the comorbidities were immune disorders. Immune-mediated destruction of the adrenal gland in humans is commonly associated with other immune disorders.[2] Polyglandular autoimmune disease is rare in dogs, with HA and hypothyroidism being the most common concurrent disorders.[2] Hypothyroidism was diagnosed in 2/19 (10%) dogs with EEH, and the most common comorbidity was primary inflammatory enteropathy. However, it is difficult to determine whether signs of gastrointestinal disease in dogs with EEH might be related to disruptions of the epithelial barrier of the gastrointestinal tract due to cortisol deficiency or concurrent primary gastrointestinal disease. A small percentage of dogs with EEH can develop abnormalities in serum electrolyte concentrations indicative of mineralocorticoid deficiency weeks to months after diagnosis.[2,3,7] Interestingly, none of the EEH dogs developed electrolyte abnormalities over a follow-up time of 31 days to six years, but aldosterone concentrations were not measured. Therefore, one cannot be sure that the dogs enroled did not have some degree of mineralocorticoid deficiency.

The present study identified clinicopathologic features, including hematocrit, RDW, serum albumin, and an albumin/globulin ratio which were significantly different between the EEH and the non-PGA dogs. These variables could be utilized to increase the index of suspicion for EEH; however, substantial overlap was observed, indicating that gold standard adrenal function testing should be carried out in dogs with a compatible clinical presentation regardless of clinicopathological abnormalities.

The present study had some limitations, including the small sample size in the EEH and the PGA groups which could have influenced the statistical power. Data from the dogs with EEH were collected retrospectively, and the absence of some parameters may have partially biased the results. This study was not designed to investigate the HPA recovery time. Hence, the ACTHst was carried out at different times after glucocorticoid discontinuation and was not carried out at a specific predetermined time. Furthermore, the glucocorticoid dose was different for each dog in the PGA group. This might have influenced the results; however, it reflected the real condition of the clinical setting.

In conclusion, the prevalence of EEH in dogs with signs of CGD was lower than previously reported. The results of this study showed that glucocorticoid administration, even for a few days, could cause false positive results on the ACTHst. The clinical and clinicopathological variables identified in the present study could increase the index of suspicion for EEH or PGA in dogs with an unclear history of glucocorticoid administration. Since dogs with HA require lifelong treatment, it is important to measure eACTH and to repeat the ACTHst when PGA is suspected.

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4.2 | Hypothalamic-pituitary-adrenal axis recovery after intermediate-acting glucocorticoid treatment in client-owned dogs

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ABSTRACT

Background

In dogs, duration of the hypothalamic-pituitary-adrenal axis (HPA-axis) suppression after systemic glucocorticoid therapy is reported to vary from a few days to up to 7 weeks after glucocorticoid discontinuation. These data derive mainly from experimental studies regarding healthy dogs and not from animals with spontaneous disease.

Hypothesis and Objective

To determine the timeline for recovery of the HPA axis in a group of ill dogs treated with intermediate-acting glucocorticoids (IAGCs).

Animals

Twenty client-owned dogs which received IAGC for at least one week.

Methods

A single-center prospective observational study. An ACTH stimulation test, endogenous ACTH, serum biochemistry and urinalysis were performed at T0 (2-6 days after IAGC discontinuation) and then every two weeks (T1, T2, T3, etc.) until HPA-axis recovery was documented (post-ACTH cortisol>6 µg/dL).

Results

The median time of the HPA axis recovery was 3 days (2-133 days). 11/20 dogs showed recovery of the HPA axis at T0, 6/20 at T1, 1 dog each at T2, T5, and T9. Dose and duration of treatment were not correlated with the timing of HPA axis recovery. ALT and ALP were significantly correlated with the post-ACTH cortisol (rs=-.34,P=.029;rs=-.31,P=.049). Endogenous ACTH was significantly correlated with pre (r=.72;P<.0001) and post-ACTH cortisol concentrations (r=.35;P=.02). The timing of HPA axis recovery of the dogs undergoing an alternate-day tapering process was not different compared to dogs which did not (3.5 vs. 3 days, P=.89).

Conclusion and clinical importance

The majority of dogs showed recovery of the HPA axis within a few days after IAGC discontinuation. However, 2/20 dogs required more than 8 weeks.

INTRODUCTION

Hypoadrenocorticism (HA) is an uncommon disease in dogs.1 Dogs with HA are frequently presented with chronic non-specific clinical signs, including anorexia, vomiting, weight loss, and diarrhea.[2-5] Due to the vague clinical signs, dogs with HA and, in particular, those with eunatremic, eukaemic HA (EEH), often receive empirical treatment with glucocorticoids (GCs) before reaching a final diagnosis. The use of GCs results in the suppression of endogenous hypothalamic-pituitary-adrenal (HPA) axis function by exerting negative feedback effects at the pituitary and hypothalamus levels.[6] The ACTH stimulation test (ACTHst) remains the gold standard for HA diagnosis.[3] However, previous GC administration can give false positive results to the ACTHst, resulting in a misdiagnosis of HA.[3] For this reason, dogs with HA, in particular EEH, represent a diagnostic challenge. Currently, no guidelines exist regarding the required time span until the ACTHst can be carried out after a dog has been treated with different GC formulations. Generally, the degree and duration of suppression of the HPA axis depends on the dose, potency, half-life, and duration of the GC treatment.[6] However, in human medicine, it has been demonstrated that the duration and severity of HPA suppression cannot be reliably predicted by dose, duration or type of GC therapy.[7,8] There are few and limited published studies regarding the duration of HPA axis suppression in dogs receiving systemic GCs. In these studies, HPA axis recovery in dogs treated with systemic GCs is reported to range from a few days to up to seven weeks after GC discontinuation.[9-15] However, the majority of these studies were carried out on healthy experimental dogs and, as such, the possible interference on HPA-axis from concurrent diseases has not been investigated. Moreover, in clinical practice, gradual tapering of the GC dose is recommended if the therapy lasts for two weeks or longer, or if high doses are used.[6] The effect of alternate-day therapy on HPA axis recovery in a clinical context has never been investigated.

The aim of this study was to determine the timeline for recovery of the HPA axis in a group of ill dogs treated with systemic intermediate-acting GCs (IAGCs). The hypothesis is that the timing of HPA-axis recovery is highly individual dependent and that dogs who underwent the alternate-day tapering process can have a more rapid recovery than dogs who did not.

MATERIAL AND METHODS

Study design

A single-center prospective observational longitudinal study involving client-owned dogs receiving systemic therapy with IAGCs (prednisone/prednisolone or methylprednisolone) which were admitted to the Veterinary Teaching Hospital of the University of Bologna from September 2020 to December 2022 was carried out. Dogs with different medical conditions (immune-mediated, neoplastic, and inflammatory) treated with IAGCs for at least one week were eligible for inclusion in the study. Only dogs in which the therapeutic protocol, in terms of dose and timing, was well defined were included. Dogs on topical GC therapy (alone or in combination with the systemic therapy) and dogs on a different type of GC therapy (e.g., dexamethasone, betamethasone) were not eligible for inclusion. All dogs were enrolled according to the study protocol which was approved by the Scientific Ethics Committee of the University of Bologna.

Animals

The following data were collected at the time of the enrolment (T0; 2-6 days after IAGC discontinuation): signalment, body weight, physical examination abnormalities, date of check-up, date of the beginning of the GC treatment, type of GC administered, therapeutic protocol used (including the dose of GC, tapering process and date of GC discontinuation), and the disease for which the dog was receiving GCs. At T0, an ACTHst, endogenous ACTH (eACTH), serum biochemistry and urinalysis including urinary protein to creatinine ratio (UPC) were carried out. Serum biochemistry, urinalysis, eACTH and ACTHst were carried out every two weeks (T1=14 days post T0, T2=28 days post T0, T3=42 days

post T0, T4=56 days post T0, etc.), until HPA axis recovery, defined as post-ACTH serum cortisol concentration >6 μg/dL (endpoint), was documented.

Endocrine testing and analytical procedures

For the ACTHst, blood samples were taken before and 60 minutes after the IV injection of 5 μg/kg synthetic ACTH (Synacthen, Alfasigma S.P.A., Bologna, Italy). Blood samples for the determination of eACTH concentrations were collected before the injection of synthetic ACTH. All the analytical procedures were carried out at the veterinary laboratory of the University of Bologna. Blood samples for the determination of the eACTH were collected into EDTA-coated plastic tubes placed on ice. The samples were immediately centrifuged at 4°C, 500g for 8 minutes, and the plasma was immediately transferred to plastic tubes, stored at 4°C and analyzed within 8 hours, or stored at -80°C and thawed immediately before analysis. Blood samples for cortisol determination were collected in serum separating tubes. The coagulated blood samples were centrifuged for 10 minutes at 3000g; the serum was immediately transferred to plastic tubes, stored at 4°C and analyzed the same day, or stored at -80°C and thawed immediately before analysis. The serum cortisol and eACTH concentrations were measured using a chemiluminescent enzyme immunoassay (Immulite 2000, Siemens Healthcare) which had been validated for dogs and is widely used in laboratories throughout the world.[20,21] A chemistry profile (AU 480, Beckman Coulter/Olympus, Brea, CA) and urinalyses were carried out using standard laboratory methods at the medical laboratory of the referral institution.

Statistical Analysis

Statistical analysis was carried out using commercial statistical software packages (GraphPad Prism 7, San Diego, California). Descriptive statistics were generated to characterize the study population. Continuous variables were presented as mean ± standard deviation (SD), or median and range (minimum and maximum value), depending on whether the data were normally or not normally distributed, respectively. The categorical variables were described with frequencies, proportions or percentages. Cumulative, maximum and median/mean daily GC dose and overall duration of treatment were extrapolated from the therapeutic protocol of each dog. The time of HPA axis recovery was calculated as the interval between the last GC administration and a post-ACTH serum cortisol concentration >6 mg/dL. The correlations between the timing required for HPA axis recovery and cumulative dose, maximum dose, median daily dose, duration of treatment and body weight was evaluated using Spearman's rank correlation coefficient (Rs). The same statistical analysis was used to investigate the correlation between post-ACTH cortisol and clinico-pathological abnormalities due to GC treatment (alanine aminotransferase, ALT; alkaline phosphatase, ALP; gamma-glutamyl transpeptidase, GGT; haptoglobin; cholesterol; triglycerides; urine specific gravity, USG; UPC) as well as between pre- and post-ACTH cortisol concentration and eACTH. Comparison between the timing required for HPA axis recovery in dogs which did and those which did not undergo the alternate-day tapering process was carried out using the Mann Whitney or the T-test. The level of significance was set at $P<.05$.

RESULTS

Animals

A total of 23 dogs were included in the study. Of them, two dogs were excluded since they were diagnosed with primary EEH. In particular, the diagnosis of EEH was based on the presence of compatible clinical signs (e.g., lethargy, hyporexia, diarrhea) coupled with 1) a persistent post-ACTH serum cortisol concentration <2 μg/dL (<55.0 nmol/l); 2) high (>58 pg/ml) plasma eACTH concentrations and 3) the absence of electrolyte abnormalities (Table 1 and Figure 1). Moreover, one additional dog was excluded owing to immune mediated hemolytic anemia (IMHA) relapse and the necessity of

reintroducing GC treatment. The final study population included 13 females, of which 10 were spayed, and 7 males of which 3 were neutered.

TABLE 1 (A) and (B) Results of the ACTH stimulation test and endogenous ACTH concentration in the two dogs diagnosed with eunatremic eukalemic hypoadrenocorticism.

Time points	Pre-ACTH cortisol (µg/dL)	Post-ACTH cortisol (µg/dL)	eACTH (pg/mL)
(A)			
T0	0.30	0.30	95
T1	0.39	0.44	300
T2	0.71	0.84	246
T3	0.84	0.87	184
T4	1.31	1.29	267
T5	1.70	1.99	193
T6	1.34	2.15	122
T7	1.54	2.06	156
T8	1.51	2.72	146
T9	1.10	1.96	143
T10	0.86	1.13	238
T12	0.53	0.60	780
T13	0.43	0.37	843
(B)			
T0	0.30	0.30	29
T1	0.30	0.30	196
T2	0.30	0.30	456
T3	0.30	0.30	760
T4	0.30	0.30	755
T5	0.30	0.30	661
T6	0.30	0.30	967

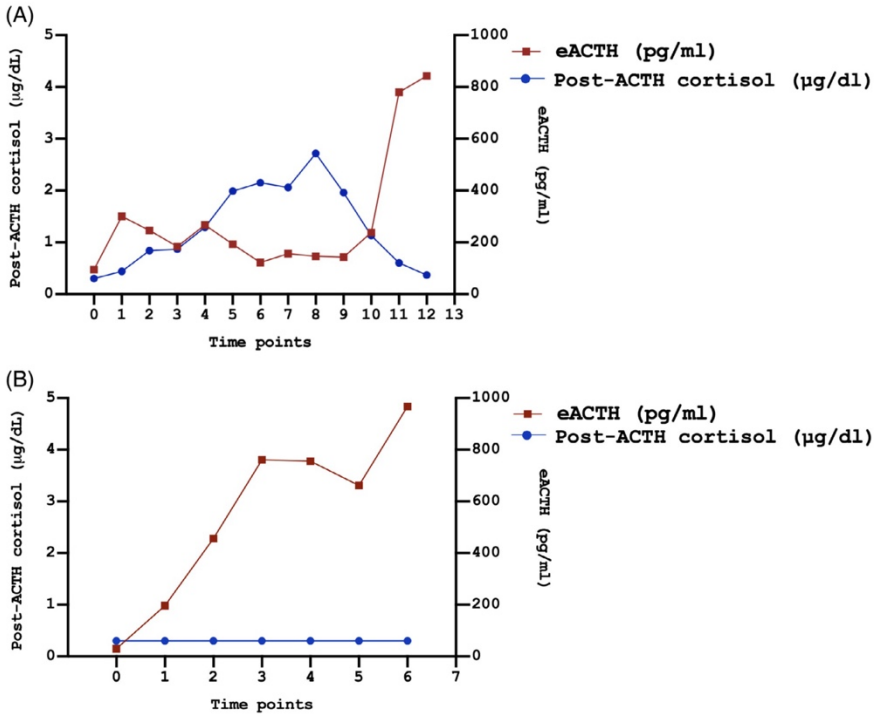

FIGURE 1 (A) and (B) serum post-ACTH cortisol concentration and endogenous ACTH concentration during the different time points in the two dogs diagnosed with eunatremic, eukalemic hypoadrenocorticism.

The median (range) age was 8.25 years (5 months-11.75 years) and the median body weight was 22 kg (4.5-44 kg). The breeds included mix-breeds (8), German Shepherds (2), Border Collies (2), American Staffordshire Terriers (2), Cocker Spaniels (1), Springer Spaniels (1), Maltese (1), Maremma Sheepdogs (1), Doberman Pinschers (1) and Spanish Greyhounds

(1). The dogs had been treated with IAGCs for the following medical conditions: IMHA (7); immunosuppressant-responsive enteropathy (3); immune-mediated polyarthritis (3); mast cell neoplasia (2); immune mediated thrombocytopenia (1); meningoencephalitis of unknown origin (1); sterile steroid-responsive lymphadenitis (1); suspicion of atypical hypoadrenocorticism (1) and protein-losing enteropathy (1). The most commonly used GC preparation was prednisolone in 16 out of the 20 dogs, followed by methylprednisolone in 4/20 dogs. The therapeutic protocol used for each single case is reported in Table 2. Table 3 shows the IAGC dose each case was receiving during the last 14 days of treatment before T0. The median cumulative dose was 58.5 mg/kg (14.7-370.5). The median maximum dose was 1.25 mg/kg/day (0.2-4). The median of the mean daily dose was 0.7 mg/kg/day (0.1-1.9). The median duration of the GC treatment was 65 days (35-534).

Timing of the HPA axis recovery

The median time of the HPA axis recovery was 3 days (2-133 days). In particular, 11/20 dogs showed recovery of the HPA axis at T0, 6/20 at T1, 1 dog each at T2, T5, and T9 (Figure 2).

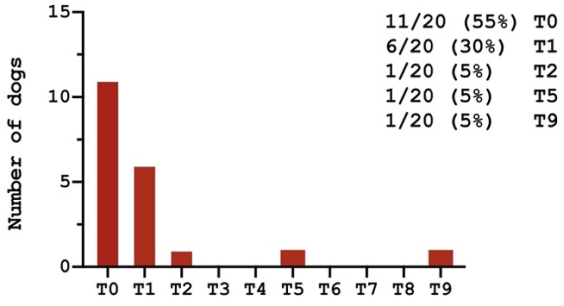

FIGURE 2 Barplot representing the percentage distribution of the population based on time of hypothalamic-pituitary-adrenal axis recovery.

The pre and post-ACTH cortisol concentration and the respective eACTH concentration in all dogs at each time-point are reported in table 4. One dog (case 11) showed undetectable (<0.3 µg/dL) pre- and post-ACTH cortisol concentrations up to T5. At T6, the eACTH became elevated (696 pg/mL) and, concurrently, the pre- and post-ACTH cortisol concentrations were detectable (2.89 and 3.26 µg/dL) for the first time. This dog reached the endpoint of the study after 4 months of GC discontinuation (T9) (Figure 3). Thirteen dogs underwent an alternate-day tapering process and 7 dogs did not. The timing of the HPA axis recovery in the dogs which underwent the alternate-day tapering process (3.5 days) was not different as compared to the dogs which did not (3 days) (P=.89).

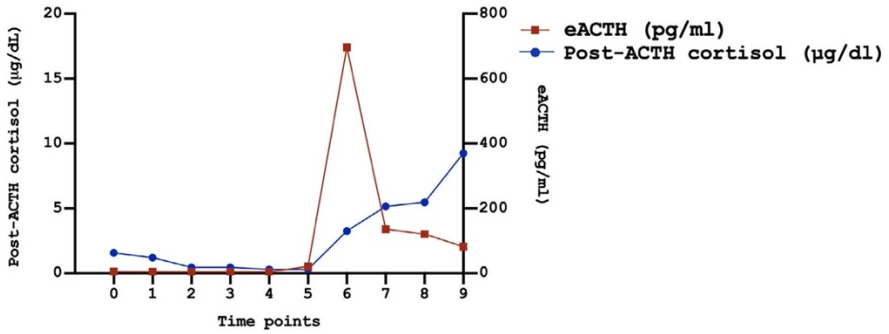

FIGURE 3 Post-ACTH cortisol concentration and endogenous ACTH concentration during the different time points in Case 11.

Correlation analysis

Cumulative dose, maximum dose, median daily dose, duration of treatment and body weight were not correlated with the timing of HPA axis recovery (Table 2).

Variables	Rs	(95% confidence interval)	*P* values
Cumulative dose	0.062	−0.501 to 0.402	.79
Maximum dose	0.042	−0.419 to 0.486	.85
Median daily dose	0.010	−0.445 to 0.461	.96
Duration of treatment	0.008	−0.460 to 0.447	.97
Body weight	−0.185	−0.589 to 0.293	.43

Note: None of the variables were correlated with the timing of HPA axis recovery.
Abbreviation: Rs, Spearman's correlation coefficient.

TABLE 2 The correlations between the timing of hypothalamic-pituitary-adrenal (HPA) axis recovery and cumulative dose, maximum dose, median daily dose, duration of treatment, and body weight.

Of the clinico-pathological abnormalities due to GC treatment, ALT and ALP were significantly negatively correlated with the post-ACTH cortisol concentration (rs=-.34, P=.029; rs=-.31, P=.049) (Figure 4). Haptoglobin, GGT, cholesterol, triglycerides, UPC and USG were not correlated with the post-ACTH cortisol (Figure 4). Endogenous ACTH was significantly positively correlated with pre (r=.72; P<.0001) and post-ACTH cortisol concentrations (r=.35; P=.02) (Figure 5 A and B).

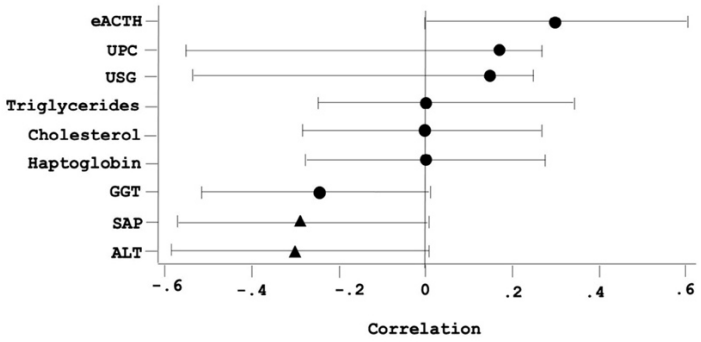

FIGURE 4 Correlations of clinical-pathological abnormalities because of glucocorticoids treatment with post-ACTH cortisol concentrations. The triangles represent a statistically significant correlation and the circles represent no significant correlation. ALP, alkaline phosphatase; ALT, alanine aminotransferase; eACTH, endogenous ACTH; GGT, gamma-glutamyl transpeptidase; UPC, urine protein: creatinine ratio; USG, urine specific gravity.

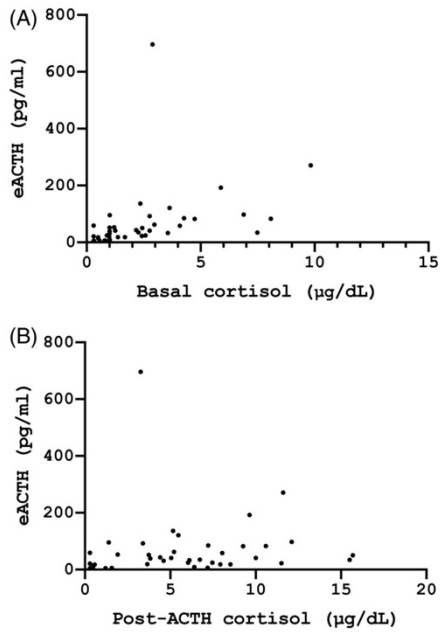

FIGURE 5 (A) The correlation between basal cortisol and endogenous ACTH (eACTH) concentration. Basal cortisol was positively correlated with eACTH (rs = 0.72; *P* < .0001). (B) The correlation between post-ACTH cortisol and eACTH concentration. Post-ACTH cortisol was positively correlated with eACTH (rs = 0.36; *P* = .02).

DISCUSSION

The results of this preliminary study showed that, in dogs treated with systemic IAGC for at least 7 days, the median time of HPA axis recovery was 3 days. Approximately half of the dogs (11/20) showed a complete recovery of the HPA axis within a few days after IAGC discontinuation. However, two out of twenty dogs required more than 8 weeks to achieve complete HPA axis recovery. These data add important information to the current literature which was based on limited studies carried out mainly on healthy research dogs receiving different types of GC preparations. In dogs, a single dose of

methylprednisolone acetate (2.5 mg/kg IM) has been shown to suppress the HPA axis for up to 7 weeks.[10,12] A single dose of triamcinolone acetonide suppressed the HPA axis for 2 to 4 weeks[11] whereas a single dose of dexamethasone resulted in a reduced cortisol response after an ACTHst for up to 32 hours.[13] In contrast, prednisone, given at a single dose of 2.2 mg/kg IM, did not result in adrenocortical suppression.[11] However, even physiological doses of prednisone/prednisolone can suppress the HPA axis when given for a prolonged time.[6] In one study, the oral administration of prednisone at 0.55 mg/kg q12h for 35 days resulted in HPA axis suppression for up to 2 weeks after prednisone discontinuation.[14] In general, all synthetic GCs suppress the corticotropin-releasing hormone and ACTH secretion; however, their effects are not equivalent. In this study, only dogs receiving IAGCs were included since these drugs are the oral GC medication most commonly used to treat chronic diseases in dogs. Looking only at the previous studies in which IAGC (prednisolone and methylprednisolone) were used, the maximum time of HPA axis recovery was 2 weeks.[14] The results of the present study are, for the majority of the dogs, comparable with those obtained in healthy experimental dogs in which IAGCs were administered. Indeed, 17/20 dogs showed complete HPA axis recovery within approximately 2 weeks after IAGC discontinuation. However, 2/20 dogs took more than 8 weeks to show complete HPA axis recovery. In particular, one of them (dog 11) showed complete HPA axis recovery 18 weeks after IAGC discontinuation. This interval was much longer as compared to the maximum time previously reported in the veterinary literature using IAGCs or any type of GC. Recovery of the HPA axis after a single administration of methylprednisolone acetate required up to 7 weeks;12 however, the latter is a long-acting depot preparation of GCs, and a longer duration of HPA axis suppression is expected as compared to IAGCs. Particularly interesting was the pre- and post-ACTH cortisol and eACTH trend at the different time points in patient 11 (Figure 3) which took 18 weeks before reaching the endpoint of the study. This dog showed undetectable pre- and post-ACTH cortisol concentrations up to T5. At the same time point, the eACTH was detectable (20 pg/mL) for the first time. At the following time point (T6), the eACTH was very high (696 pg/mL), and the results of the ACTHst showed a subnormal response to ACTH stimulation (pre-ACTH cortisol=2.9 µg /dL and post-ACTH cortisol =3.3 µg /dL). Looking at these results, a misdiagnosis of HA in a dog with potentially compatible clinical signs is possible. Therefore, it is necessary to consider that following GC treatment discontinuation, some dogs may require a long HPA axis recovery time. To confirm the presence of HA in these cases, sequential ACTHsts and eACTH measurements might be needed.

In this study, all the dogs underwent a progressive tapering of the IAGC dose, and 13 dogs out of the 20 underwent the alternate-day tapering process. The latter should allow the HPA axis to recover on the “off-days” and is assumed to provide more rapid HPA axis recovery. However, according to the present results, the timing of HPA axis recovery in dogs which underwent an alternate-day tapering process (3.5 days) was comparable to the dogs which did not (3 days). These results suggest that a gradual decrease in the GC dose, even without an alternate-day tapering process, might allow rapid recovery of the HPA-axis. Additional studies are needed to assess whether the alternate day-tapering process affects HPA axis recovery time. It is stated that the length of time required for full axis recovery depends on the duration, dose, preparation, and frequency of application of the GCs.[6] In this study, no correlations between the timing of HPA axis recovery and cumulative dose, maximum dose, median daily dose and duration of treatment were found. Other studies in humans had similar findings.[16-19] Therefore, according to these results, the dose and the duration of treatment do not seem to affect the timing of HPA axis recovery. However, the small sample size could have caused a type II statistical error. In support of this, the two dogs (case 3 and patient 11) which took the longest time to show a complete recovery of the HPA axis had received the longest duration of treatment and the highest median daily dose.

Anecdotally, and based on a recent study,[20] dogs with higher body weight experience a higher incidence of adverse effects as compared to dogs with lower body weights when receiving GC treatment. Considering this aspect, the Authors wanted to investigate the correlation between the timing of HPA axis recovery and body weight. According to the present

results, the timing of HPA axis recovery was not significantly correlated with body weight. Once again, the lack of statistical significance might be due to the small sample size.

The elevation of liver enzymes is among the most common biochemical abnormalities in dogs receiving GC treatment.[6] In this study, ALT and ALP concentrations were significantly negatively correlated with the post-ACTH cortisol concentration; in contrast GGT concentration was not. This finding can be explained by the fact that after GC treatment is discontinued, the liver enzymes progressively decrease and return to baseline. At the same time, the post-ACTH cortisol concentration increases due to the progressive recovery of the HPA-axis. The lack of statistical significance for GGT might be due to the less consistent effect of GC treatment on GGT as compared to the ALP and ALT.[21-27]

Endogenous ACTH was significantly positively correlated with both pre-and post-ACTH cortisol concentrations. The use of GCs results in the suppression of the endogenous HPA axis function by exerting negative feedback effects on the pituitary gland and hypothalamus. After discontinuing GC treatment, the negative feedback induced by the exogenous GC administration decreases, resulting in a progressive increase in eACTH and, at the same time, a progressive increase in basal and post-ACTH cortisol concentrations.

An interesting and unexpected finding of this study was that during case recruitment, 2 dogs were excluded from the final analysis because they had been diagnosed with EEH. The diagnosis of EEH was based on the presence of compatible clinical signs (e.g., lethargy, hyporexia, diarrhea) coupled with 1) persistent post-ACTH serum cortisol concentration <2 µg/dL (<55.0 nmol/L); 2) high (>58 pg/mL) plasma eACTH concentrations and 3) the absence of electrolyte abnormalities. In these dogs, GC treatment was discontinued 12 and 16 weeks before the EEH diagnosis. Both dogs had received prednisolone for the treatment of IMHA. The high occurrence of EEH in this population of dogs might reflect a common aetiopathogenesis for both HA and IMHA. Indeed, IMHA involves autoimmunity to self-antigens on the erythrocyte cell membrane.[28] Several facts provide strong evidence for HA also being an immune-mediated condition.[29-31] Polyglandular endocrine disease has been reported in veterinary medicine.[32-37] Up to 2.3% of dogs diagnosed with endocrine disease are diagnosed with multiple endocrinopathies.[38] However, concurrent non-endocrine autoimmune disorders have only rarely been reported.[39-41] The occurrence of multiple immune-mediated diseases might be coincidental or reflective of a common aetiopathogenesis, though the latter is often considered to be likely owing to an underlying predisposition (genetic, environmental) or an immune trigger (infective, neoplastic, drug/toxin). Thus, the presence of one endocrine automimmune disorder should alert clinicians to the possibility of a patient developing concurrent immune-mediated diseases.

The present study had some limitations. First, the small sample size might have decreased the statistical power, leading to type II errors. Second, the dogs included in the study underwent different therapeutic protocols in terms of dosage and duration of treatment. Third, the majority of dogs received IAGCs for immune-mediated diseases, which are associated with higher doses of GCs as compared to the doses usually received by dogs with suspected HA. This may have influenced the result; however, it might reflect the real condition of the clinical setting.

In conclusion, the optimal time to test for HPA axis recovery following prolonged GC use remains controversial due to the variability of data regarding the timelines of when that occurs. Clinicians should be aware that, after IAGC treatment for a prolonged period, the earliest that HPA axis recovery may be seen is approximately 2 to 6 days after GC discontinuation. However, some dogs can require more than 8 weeks. This extended time period could cause false positive results on the ACTHst, resulting in a misdiagnosis of HA.

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4.3 | Urinary cortisol-creatinine ratio in dogs with hypoadrenocorticism

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ABSTRACT

Background

Basal serum cortisol (BSC) ≥2 mg/dL (>55 nmol/L) has high sensitivity but low specificity for hypoadrenocorticism (HA).

Objective

To determine whether the urinary corticoid:creatinine ratio (UCCR) can be used to differentiate dogs with HA from healthy dogs and those with diseases mimicking hypoadrenocorticism (DMHA).

Animals

Nineteen healthy dogs, 18 dogs with DMHA, and 10 dogs with HA.

Methods

Retrospective study. The UCCR was determined on urine samples from healthy dogs, dogs with DMHA, and dogs with HA. The diagnostic performance of the UCCR was assessed based on receiver operating characteristics (ROC) curves, calculating the area under the ROC curve.

Results

The UCCR was significantly lower in dogs with HA (2.03 x 10^{-6}; range 1.04-3.81 x 10^{-6}) as compared to healthy dogs (10.55 x 10^{-6} ; range 3.47-54.05 x 10^{-6}) and those with DMHA (32.09 x 10^{-6}; range 7.68-245.5 x 10^{-6}) (P <.0001). There was no overlap between dogs with HA and dogs with DMHA. In contrast, one healthy dog had a UCCR value in the range of dogs with HA. The area under the ROC curve was 0.99. A cut-off value of UCCR <4.4 yelded 100% sensitivity and 97.3% specificity in diagnosing HA.

Conclusions and Clinical Importance

The UCCR seems to be a valuable and reliable screening test for HA in dogs. The greatest advantage of this test is the need for only a single urine sample.

INTRODUCTION

Hypoadrenocorticism (HA) is the umbrella term for a range of naturally-occurring or iatrogenic disorders which cause a reduced function of the adrenal cortex and results in a state of glucocorticoid deficiency, mineralocorticoid deficiency or both.[1] In dogs, the majority of cases of naturally occurring HA result from primary adrenal gland failure which is thought to be a result of the immune-mediated destruction of the adrenal cortices.[2,3]

Dogs with HA are frequently presented with vague, episodic and nonspecific clinical signs, including anorexia, vomiting, weight loss, and diarrhea.[2-5] The most common biochemical abnormalities include azotemia and electrolyte abnormalities, such as hyponatremia, hyperkalemia and a low-sodium to-potassium ratio. However, up to 30% of dogs with HA have what has been defined eunatremic eukalemic HA, where electrolyte concentrations remain within the reference range.[4,6-9] The absence of typical electrolyte abnormalities makes eunatremic eukalemic HA more difficult to suspect and diagnose in a clinical setting. On the other hand, signs of gastrointestinal disease secondary to a lack of glucocorticoids are indistinguishable from clinical signs caused by primary gastrointestinal disorders.[3,10-12]

A definitive diagnosis of HA requires an ACTH stimulation test (ACTHST).[2,3] However, the high cost and limited availability of synthetic ACTH in some countries, coupled with the requirement for repeated venipuncture, are some limitations of this test. Cortisol-to-ACTH ratio also revealed a valuable tool for the diagnosis of primary HA with the greatest advantage of a single blood sample needed.[9,13,14] However, the main limitation of measurement of plasma ACTH in practice is the instability of the hormone. To avoid degradation blood must be collected in precooled Ethylene Diamine Tetra Acetic Acid plastic tubes, processed immediately, chilled, and frozen until analysis. This procedure is time-consuming and cost-intensive. For this reason, basal serum cortisol (BSC) concentration, an easier and cheaper screening diagnostic test, is routinely used in dogs with suspicion of HA. Using a cut-off value of ≥2 mg/dL (>55 nmol/L), the negative predictive value is reported to be between 99.8 to 100%.[15-17] However, the specificity of the test varies from 20 to 78.2%.[14-16] Therefore, due to the low specificity of the test, an ACTHST must be performed in dogs with BSC ≤2 mg/dL (≤ 55 nmol/L) to exclude HA. Since up to 33% of dogs with signs of chronic gastrointestinal disease, but without HA, which are those most commonly screened for HA in clinical practice, have an BSC <2 mg/dL (<55 nmol/L),[17,18] this means that the ACTHST must often be carried out to exclude HA, with a consequent increase in the diagnostic costs and time for the client.

The urinary corticoid:creatinine ratio (UCCR) provides an integrated measurement of corticoid production over a given interval, thereby overcoming the problem of fluctuations in plasma concentrations.[19] The greatest advantage is the need for only a single urine sample. Moreover, it is easy to carry out and relatively economical. The UCCR is currently routinely used as a screening test for dogs with spontaneous hypercortisolism,[20] and few studies have investigated its performance in monitoring dogs with hypercortisolism on a trilostane or mitotane regimen.[21-26] However, the use of the UCCR has not been evaluated in diagnosing spontaneous HA.

The aim of this study was to determine whether the UCCR could be used to differentiate dogs with HA from normal dogs and those with diseases mimicking HA (DMHA). Our hypothesis was that the UCCR would prove to have a diagnostic value in differentiating dogs with HA from dogs with DMHA.

MATERIALS AND METHODS

Animals and study design

Urine samples stored at −20°C from privately owned dogs were retrospectively selected from the University of Bologna Veterinary Teaching Hospital digital database. The urine samples had been collected from June 2019 to February 2021 from dogs with HA or DMHA at the time of diagnosis, and at routine check-ups from the healthy dogs. As per Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010, regarding the protection of

animals used for scientific purposes, the Italian legislature (D. Lgs. n. 26/2014) does not require approval from ethical committees for the use of stored samples in retrospective studies.

Dogs were included in the HA group if the post-ACTH serum cortisol was ≤2 mg/dL (≤55 nmol/L), and a clinical diagnosis of naturally occurring HA was made. Dogs were excluded from the study if a glucocorticoid medication had been administered within 90 days before testing. Other dogs for which HA was suspected on the basis of clinical signs (vomiting, diarrhea, weakness, lethargy) but was subsequently excluded based on the BSC >2 mg/dL (>55 nmol/L) or ACTHST results (post-ACTH serum cortisol >5 mg/dL[>138 nmol/L])[27] were included in the DMHA group. Dogs were defined as healthy if no abnormal clinical signs were reported and if hematology, serum biochemistry and urinalysis results were within the reference intervals.

Sample collection and endocrine tests

For the ACTHST, blood samples were taken before and 60 min after the IV injection of 5 mg/kg synthetic ACTH (Synacthen, Alfasigma S.P.A., Bologna, Italy). Blood samples for the determination of cortisol were collected in serum separating tubes. Coagulated blood samples were centrifuged for 10 minutes at 3,000 g; the serum was immediately transferred to plastic tubes, stored at 4°C and analyzed the same day, or stored at −20°C and thawed immediately before analysis. For the UCCR determination, stored urine samples were thawed at room temperature and immediately analyzed to measure creatinine and cortisol urine concentration. The urine samples were collected by free-catch (at home or in the hospital) or by US-guided cystocentesis. The UCCR was measured on the same day for all 3 groups of dogs (healthy, HA, DMHA).

Analytical procedures

Serum cortisol and urine cortisol concentrations were measured with a chemiluminescent enzyme immunoassay using the antibody poll before kit lot 55026 (Immulite 2000, Siemens Healthcare) which had been validated for dogs and is widely used in laboratories throughout the world.28 Urine creatinine concentrations were measured using an automatic analyzer (AU480, Beckman Coulter/Olympus, Brea, California, USA). The UCCR was calculated by dividing the urine cortisol concentration (nmol/L) by the urine creatinine concentration (mmol/L).

Statistical analysis

Statistical analysis was carried out using commercial statistical software packages (GraphPad Prism 7®, San Diego, California, USA). Data were presented as median and range, and analyzed by nonparametric tests. Differences between groups for categorical and numerical variables were analyzed using the Fisher's exact test and the Kruskal Wallis test, respectively. The Kruskal–Wallis test followed by Dunn's post test was carried out to compare the UCCR from dogs with HA, dogs with DMHA and healthy dogs. A receiving operating characteristic (ROC) curve was used to determine the area under the curve (AUC) and select the optimum UCCR cut-off values to diagnose or exclude HA. The ROC curve analysis was carried out by combining healthy and DMHA dogs versus HA dogs. A 95% confidence interval was calculated for the ROC curve. The level of significance was set at $P < .05$.

RESULTS

Animals

Ten dogs with HA were included. Their ages ranged from 40 to 92 months (median, 60.5 months) and their body weights from 3.7 to 39.6 kg (median, 13.2 kg). There were 4 males (3 castrated) and 6 females (5 spayed). The HA group consisted of 6 purebred dogs (2 Jack Russell Terriers, 1 English Setter, 1 Cocker Spaniel, 1 Rottweiler, 1 Miniature Pinscher) and 4 mixed breed dogs. All the dogs were diagnosed with primary HA. Only one dog had primary eunatremic eukalemic HA.

Eighteen dogs with DMHA were included. Their ages ranged from 8 to 147 months (median, 48 months) and their body weights from 5 to 53.5 kg (median, 24.1 kg). There were 12 males (2 castrated) and 6 females (3 spayed). This group consisted of 13 purebred dogs (2 Golden Retrievers, 1 Labrador Retriever, 2 Jack Russell Terriers, 2 Poodles, 1 Dog de Bordeaux, 1 Bernese Mountain dog, 1 French Bulldog, 1 Cavalier King Charles Spaniel, 1 Great Dane, and 1 Vizsla) and 5 mixed breed dogs. The final diagnoses were chronic gastroenteritis (12), acute gastroenteritis (4), pancreatitis (1) and adrenal neoplasia (1).

Nineteen healthy dogs were included. Their ages ranged from 13 to 98 months (median, 61 months) and their bodyweights from 6.5 to 32.0 kg (median, 23 kg). There were 7 males (4 castrated) and 12 females (8 spayed). This group consisted of 10 mixed breed dogs and 9 purebred dogs (2 Border Collie, 1 Boxer, 1 Cavalier King Charles Spaniel, 1 Lagotto Romganolo, 1 Jack Russell Terrier, 1 Spanish Greyhound, 1 Labrador Retriever and 1 Cane Corso. There were no significant differences between groups for age, sex and body weight.

Twelve out of the 18 (66 %) dogs with DMHA had BSC ≤2 mg/dL (<55 nmol/L). In these dogs, HA was excluded on the basis of post-ACTH serum cortisol >5 mg/dL [>138 nmol/L]). The remaining 6 dogs had BSC > 2 mg/dL (>55 nmol/L); therefore, no additional tests were needed.

UCCR

The median UCCR was 2.03 x 10^{-6} (1.04-3.81 x 10^{-6}), 32.09 x 10^{-6} (7.68-245.5 x 10^{-6}), and 10.55 x 10^{-6} (3.47-54.05 x 10^{-6}) in dogs with HA, dogs with DMHA and healthy dogs, respectively. The median UCCR was significantly lower (P<0.0001) in the dogs with HA as compared to the dogs with DMHA and the healthy dogs (Figure 1). There was no overlap between dogs with HA and dogs with DMHA. In contrast, one healthy dog had a UCCR value in the range of dogs with HA (Figure 1). The UCCR was significantly higher in dogs with DMHA as compared to healthy dogs (P=0.013) (Figure 1). The median UCCR in dogs with DMHA and BSC ≤2 µg/dL (≤55 nmol/L) was 27.14 x 10^{-6} (7.68-245.46 x 10^{-6}). The area under the ROC curve was 0.99 (95% CI: 0.98-1.00). A cut-off value of UCCR < 4.4 revealed 100% sensitivity (95% CI: 69.1-100) and 97.3% specificity (95% CI: 85.8-99.9) in diagnosing HA.

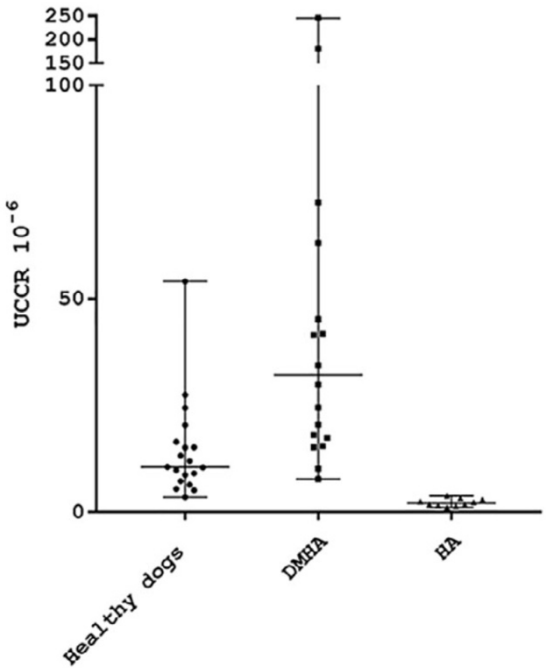

FIGURE 1 Scatter scale plot comparing urinary corticoid:creatinine ratio (UCCR) of dogs with hypoadrenocorticism (HA, n = 10), dogs with disease mimicking hypoadrenocorticism (DMHA, n = 18) and healthy dogs (healthy, n = 19). The horizontal bars represent the median, the maximum, and the minimum value of each group

DISCUSSION

The results of this study show that the dogs with HA had a significantly lower UCCR than the healthy dogs and dogs with DMHA. A UCCR value >4.4 x 10^{-6} could be useful in excluding HA in dogs since the sensitivity of the test using this cut-off was 100%. Using the same cut-off value, the specificity of the test was 97.3%. None of the dogs with DMHA had a UCCR <4.4 x 10^{-6} (the lowest UCCR value detected was 7.68 x 10^{-6}). However, 1 healthy dog had a UCCR value <4.4 x 10-6 (UCCR=3.47 x 10^{-6}).

Basal serum cortisol concentration is currently routinely used as a screening test for HA in dogs due to the evidence that BSC >2 µg/dL (>55 nmoL/L) is 100% sensitive for excluding HA.[16] However, the specificity of the test for the same cut-off is low and varies from 20 to 78.2%.[14-16] The specificity of the BSC is higher if using a cut-off < 1 µg/dL (28 nmol/L) and vary from 91.5 to 98.2%.[15-17] However, the sensitivity of the test for this cut-off decreases up to 85.7%,[16] resulting in an increased number of false negatives. There are potentially serious consequences of missing a diagnosis of HA. Therefore, currently, the use of the higher cut-off >2 µg/dL (>55 nmoL/L) is advocated to exclude the disease. According to our results, the specificity of the UCCR was higher (97.3%) than the specificity of BSC. Furthermore, considering only dogs with DMHA, which are those routinely screened for HA in the clinical practice, the specificity of the test was 100%. The higher specificity of the UCCR as compared to BSC in detecting HA could be explained by the normal episodic secretion of cortisol in dogs. In this species, cortisol concentrations can become intermittently low or undetectable over a 24-hour period.[29,30] In contrast, the UCCR provides a measurement of corticoid production over a period of several hours, thereby overcoming the problem of fluctuations in plasma concentrations.[19] Although these results require confirmation by large-scale studies, the UCCR might allow a clearer differentiation between dogs with HA and dogs with DMHA. Therefore, the use of the UCCR could be an alternative screening test for HA, thus reducing costs for the owners. In addition, measuring the UCCR is less time-consuming and less invasive for the animal.

In humans, measuring urinary free cortisol levels has a low diagnostic sensitivity in detecting HA since approximately 20% of people with adrenal insufficiency have normal values.[31,32] Therefore, it is not considered a valid test for the diagnosis of HA in humans.[33] The low sensitivity of the test could be related to the severity of adrenal insufficiency with lower cortisol urinary excretion in the case of more severe adrenal insufficiency and low-normal results in patients with partial adrenal insufficiency.[34] This discrepancy between the present results and those reported in human medicine requires confirmation by large-scale studies. However, a possible explanation could be the degree of adrenal insufficiency which, in veterinary patients, could be more severe at the time of diagnosis as compared to human patients in whom the clinical signs of adrenal insufficiency are more likely to be recognized earlier compared to veterinary patients.

Up to 30% of dogs with HA have what has been called eunatremic eukalemic HA where serum electrolyte concentrations are normal at the time of diagnosis.[4,6,7,9] This subset of dogs might be more likely to undergo screening tests as opposed to a complete ACTHST given the lower index of suspicion of disease. As such, it is important to consider the diagnostic performance of UCCR in both subsets (with normal and abnormal electrolytes) of dogs. Only one dog included in the present study had eunatremic eukalemic HA and its UCCR was similar to the values obtained in dogs with hyponatremic and/or hyperkalemic HA. However, the diagnostic utility of the UCCR in dogs with and without electrolyte abnormalities should be additionally investigated. In this study, only dogs with spontaneous HA have been included. Dogs with iatrogenic HA receiving glucocorticoids that do not cross-react with cortisol assay, such as dexamethasone, might have a value of UCCR overlapping with those of dogs with spontaneous HA. If so, a complete and detailed clinical history would be necessary to distinguish between dogs with spontaneous and iatrogenic HA. However, to confirm this hypothesis, further studies are needed.

Measurement of cortisol-to-ACTH ratio is an alternative valuable and reliable tool for the diagnosis of primary hypoadrenocorticism in dogs.[9,13,14] Similar to the UCCR, it allows to discriminate between dogs with HA and those with DMHA with a sensitivity of 100% and a specificity of 99%.[14] However, the diagnostic utility of this test is limited in clinical practice because of the critical sampling collection and handling needed for the ACTH measurement. Moreover, the cortisol-to-ACTH ratio might be less useful compared to the UCCR in dogs with secondary HA.

The present study had several limitations. First, the small number of dogs included in each group could have markedly affected the calculated sensitivities and specificities of the UCCR to detect HA in dogs. Unfortunately, the number of dogs included in each group was limited since, few days after the analysis of the samples, there was a change in the Immulite 2000 antibody used for cortisol measurement. An initial review by the European Society of Veterinary Endocrinology - Endocrine Quality Assurance, based on >40 canine urine results, suggested that the new kit canine urine cortisol results were lower (average bias -70%) than the values obtained with the previous kit Lot (from kit Lot 550 backward).[35] Based on the above, the use of the new assay could have resulted in greater overlap between the UCCR values of dogs with HA and those with DMHA or healthy dogs. Therefore, the UCCR cut-off established in this study might need to be validated again with the new assay. Finally, due to the retrospective nature of the study, the method of urine collection was not standardized and not recorded. Veterinary care and setting could increase the overall stress level and, consequently, the UCCR in dogs.[36,37] This could have affected the results of the present study, resulting in higher UCCR values if the urine was collected in the hospital and lower if the urine was collected at home. In this regard, the collection of urine in the hospital can offer an advantage in dogs that underwent UCCR measurement as a screening test of HA. Indeed, veterinary care and setting can induce a stress response with subsequent increased serum cortisol concentration and UCCR in dogs with DMHA but not in dogs with HA, which are not able to mount a stress response due to the adrenal gland failure.

In conclusion, the determination of the UCCR seems to be a valuable and reliable screening test for HA in dogs. Using a cut-off >4.4 x 10-6,, differentiation between dogs with HA and those with DMHA was 100%. The most significant advantage of this test was is the need for only a single urine sample.

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4.4 | Comparison of urinary cortisol, urinary cortisol to creatinine ratio, and basal serum cortisol as a screening test for hypoadrenocorticism in dogs

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ABSTRACT

Objective

This study investigates whether urinary cortisol (UC) and urinary cortisol-to-creatinine ratio (UCCR) perform better than basal serum cortisol (BSC) in identifying dogs with HA.

Methods

One-hundred-and-twenty client-owned dogs: 20 with HA, 42 healthy, and 60 with diseases mimicking HA (DMHA). Retrospective multicenter study. The UC and UCCR were determined on urine samples using a chemiluminescent enzyme immunoassay. The diagnostic performance of the UC and UCCR were assessed based on receiver operating characteristics (ROC) curves.

Results

A cut-off value of UC <2 µg/dL revealed 100% sensitivity (95% CI: 83.2-100) and 90.0% specificity (95% CI: 79.5-96.2) in diagnosing HA. A cut-off value of UCCR <8.5 x 10^{-6} revealed 100% sensitivity (95% CI: 83.1-100) and 71.7% specificity (95% CI: 58.6-82.6) in diagnosing HA. A cut-off value of BSC <2 µg/dL and <1 µg/dL revealed 100% sensitivity (95% CI: 83.2-100), 51.7% specificity (95% CI: 38.5-64.8) and 100% sensitivity (95% CI: 83.9-100), 90% specificity (95% CI: 79.8-95.3) in diagnosing HA, respectively.

Conclusions

UC and UCCR showed comparable sensitivity but better specificity than BSC <2 µg/dL in identifying dogs with HA.

Clinical Relevance

UC and UCCR should be considered promising screening tests for canine HA.

INTRODUCTION

Hypoadrenocorticism (HA) is an uncommon disease in dogs.[1] Dogs with HA are frequently presented with chronic unspecific clinical signs, including anorexia, vomiting, weight loss, and diarrhea.[2-5] Clinical signs of HA in dogs are vague, often waxing and waning, and none are pathognomonic of the disease. This is particularly true with eunatremic, eukalemic HA (EEH), also defined as "atypical" hypoadrenocorticism.[1] A definitive diagnosis of HA requires an ACTH stimulation test (ACTHst).[2] However, the high cost and limited availability of synthetic ACTH in some countries, coupled with the requirement for repeated venipuncture, are some limitations of this test. As a result, basal serum cortisol (BSC) concentration, using a cut-off value of ≥2 μg/dL, is commonly used as a screening test to rule out HA. BSC concentration <2 μg/dL has excellent sensitivity for HA (99.4%-100%).[6-8] However, due to the low specificity of the test (20%-78.2%), up to 47% of dogs with gastrointestinal signs but without HA, have a BSC <2 μg/dL.[6-12] Two recent studies investigated the urine cortisol-to-creatinine ratio (UCCR) as an alternative screening test for HA in dogs.[13,14] In the study of Del Baldo and co-authors a UCCR cut-off value of $<4.4 \times 10^{-6}$ yielded 100% sensitivity and 97.3% specificity in diagnosing HA.[13] The study of Moya et al. 2022 showed even better diagnostic performances.[14] In both studies, urinary cortisol was measured using a chemiluminescent immunoassay (CLIA) (Immulite 2000 cortisol; Siemens Health Care Diagnostics Ltd). Unfortunately, after analyzing the samples of the 2 above-mentioned studies, there was a change in the Immulite 2000 antibody used for cortisol measurement. An initial review by the European Society of Veterinary Endocrinology (ESVE)—Endocrine Quality Assurance, based on >40 canine urine results, suggested that the cortisol values measured with the new antibody were lower (average bias -70%) than the values obtained with the previous antobody (kit before Lot 550).[15] Based on the above findings, the use of the new antibody might result in different diagnostic performances. Therefore, new reference intervals and diagnostic performances should be evaluated using the currently available antibody. If the diagnostic performance of UCCR, measuring cortisol with the new antibody, remains good or similar to that observed in previous studies,[13,14] we hypothesize that UCCR and also urinary cortisol (UC), measured as an absolute value, might be better than BSC in identifying dogs with HA. This study aims to investigate whether UC and UCCR, used as screening tests, perform better than BSC in identifying dogs with HA.

MATERIAL AND METHODS

Animals and study design

Urine samples collected from privately owned dogs and stored at -20°C or -80°C were retrospectively selected from the Veterinary Teaching Hospital of the University of Bologna digital database. The urine samples were collected from January 2020 to March 2023 from dogs with HA or dogs with diseases mimicking HA (DMHA) at the time of diagnosis, and routine check-ups from the healthy dogs. The protocol was approved by the Scientific Ethics Committee of the University of Bologna (no. 57790/2023). Dogs were included in the HA group if consistent clinical and clinicopathological abnormalities were present and the post-ACTH serum cortisol was ≤2 μg/dL. A diagnosis of EEH was made if the following criteria were met: (a) post-ACTH serum cortisol concentration <2.0 μg/dL; (b) high (>58 pg/mL) or undetectable (<5 pg/mL) plasma endogenous ACTH (eACTH) concentrations, and (c) the absence of electrolyte abnormalities. Dogs were excluded from the study if a glucocorticoid medication had been administered within 90 days before testing. Other dogs for which HA was suspected based on clinical signs (vomiting, diarrhea, weakness, lethargy) but was subsequently excluded based on the BSC >2 μg/dL or ACTHst results (post-ACTH serum cortisol >5 μg/dL)[16] were included in the DMHA group. Dogs were defined as healthy if no abnormal clinical signs were reported and CBC, serum biochemistry, and urinalysis results were within the reference intervals.

Sample collection and analytical procedures

The urine samples were collected by free-catch (at home or in the hospital) or by US-guided cystocentesis performed without sedation of the dog. For the ACTHst, blood samples were taken before and 60 minutes after the IV injection of 5 µg/kg synthetic ACTH (Synacthen, Alfasigma S.P.A., Bologna, Italy). Blood samples for the determination of eACTH concentrations were collected before the injection of synthetic ACTH.

All the analytical procedures were carried out at the veterinary laboratory of the University of Bologna. Blood samples to determine the eACTH were collected into EDTA-coated plastic tubes placed on ice. The samples were immediately centrifuged at 4°C, 500g for 8 minutes, and the plasma was immediately transferred to plastic tubes, stored at 4°C, and analyzed within 8 hours, or stored at -80°C and thawed immediately before analysis. Blood samples for the cortisol determination were collected in serum-separating tubes. Clotted blood samples were centrifuged for 10 minutes at 3000g; the serum was immediately transferred to plastic tubes, stored at 4°C and analyzed the same day, or stored at -80°C and thawed immediately before analysis. The serum cortisol and eACTH concentrations were measured using a chemiluminescent enzyme immunoassay (Immulite 2000, Siemens Healthcare) validated for dogs and widely used in laboratories worldwide.[17,18] Subsequent batches of the kit Lot 550 were used for cortisol analysis. The lower limit of quantification of the assay for cortisol was 1 µg/dL. The chemistry profile and urine creatinine concentration were measured using an automatic analyzer (AU 480, Beckman Coulter/Olympus, Brea, CA). The UCCR was calculated from creatine and cortisol values as previously described.[19]

Statistical Analysis

Statistical analysis was carried out using commercial statistical software packages (GraphPad Prism 7, San Diego, California). Data were presented as median and range and analyzed by nonparametric tests. Differences between groups for categorical and numerical variables were analyzed using the Fisher's exact test and the Kruskal-Wallis test, respectively. The Kruskal-Wallis test followed by Dunn's post-test was carried out to compare the UC, the UCCR, and the BSC from dogs with HA, dogs with DMHA and healthy dogs. A receiving operating characteristic (ROC) curve was used to determine the area under the curve (AUC) and select the optimum UCCR cut-off values to diagnose or exclude HA. The ROC curve analysis was carried out by comparing HA dogs with DMHA. A 95% confidence interval was calculated for the ROC curve. Since the lower limit of quantification of the assay for cortisol was 1 µg/dL, concentrations of serum or urinary cortisol below 1 µg/dL were reported as 1 µg/dL. The level of significance was set at $P < .05$.

RESULTS

Animals

Twenty dogs with HA were included. Their age ranged from 1 to 13 years (median, 5.5 years) and their body weight ranged from 5.0 to 40.9 kg (median, 17.0 kg). There were 9 males (4 castrated) and 11 females (9 spayed). The HA group consisted of 10 purebred dogs (2 Jack Russell Terriers, 1 English Setter, 1 German Shepherd, 1 Standard Poodle, 1 Rottweiler, 1 Doberman Pinscher, 1 Abruzzese Maremma Shepherd, 1 Italian Spitz, and 1 Iberian Podenco) and 10 mixed breed dogs. Nineteen dogs had a primary HA, and 1 had a secondary HA. Six dogs had eunatremic eukalemic HA.

Sixty dogs with DMHA were included. Their age ranged from 0.5 to 15 years (median, 4 years), and their body weight ranged from 3.6 to 50.0 kg (median, 19.8 kg). There were 36 males (4 castrated) and 24 females (9 spayed). Mixed breeds (n = 11) were most common, followed by Labrador Retrievers (n = 4), Miniature Poodle (4), Maltese dog (4), German Shepherds (n = 3), Border Collies (n=3),and 31 other purebred dogs for a total of 29 different breeds. The final diagnoses were chronic enteropathy (53), acute gastroenteritis (5), and megaesophagus (2).

Forty-two healthy dogs were included. Their age ranged from 1 to 15 years (median, 4 years), and their body weight ranged from 2.8 to 49.0 kg (median, 27.0 kg). There were 18 males (5 castrated) and 24 females (12 spayed).

Mixed breeds (n = 18) were most common, followed by Labrador Retrievers (n = 5), Golden Retrievers (3), German Shepherds (n = 3), and 13 other purebred dogs for a total of 16 different breeds.

There were no significant differences between groups for age and body weight, while spayed females were more represented in the HA group and intact males in the DMHA group. In the group of dogs with HA, 14/20 had hyponatremic and/or hyperkalemic HA and 6/20 had EEH. In dogs with EEH 5 had high eACTH concentrations [median 843 pg/mL (258-1134)] and one had eACTH <5 pg/mL.

Basal Serum Cortisol

The median BSC (µg/dL) was 1.0 (1.0-1.0), and 2.1 (1.0-16.3) in dogs with HA, and dogs with DMHA, respectively. The BSC was below 2 µg/dL in 20/20 (100%), and in 28/60 (46.7%) dogs with HA, and DMHA, respectively. The BSC was significantly lower (P < .0001) in dogs with HA than in DMHA (Figure 1). The area under the ROC curve to discriminate dogs with HA from DMHA was 0.95 (95% CI: 0.90-0.99). A cut-off value of BSC <2 µg/dL revealed 100% sensitivity (95% CI: 83.2-100) and 51.1% specificity (95% CI: 38.5-64.8) in diagnosing HA. A cut-off value of BSC ≤1 µg/dL revealed 100% sensitivity (95% CI: 83.9-100) and 90.0% specificity (95% CI: 79.8-95.3) in diagnosing HA.

Urinary cortisol

The median UC (µg/dL) was 8.7 (1.0-58.5), 1.0 (1.0-1.6), and 10.4 (1.0-293.0) in HD, dogs with HA, and dogs with DMHA, respectively. The UC was below 2 µg/dL in 1/42 (2.4%) HD, 20/20 (100%) dogs with HA and 6/60 (10.0%) dogs with DMHA. The UC was significantly lower (P < .0001) in dogs with HA as compared to HD and DMHA, but there was no significant difference between HD and DMHA (Figure 2). The area under the ROC curve to discriminate dogs with HA from DMHA was 0.98 (95% CI: 0.95-1.00). A cut-off value of UC <2 µg/dL revealed 100% sensitivity (95% CI: 83.2-100) and 90.0% specificity (95% CI: 79.5-96.2) in diagnosing HA.

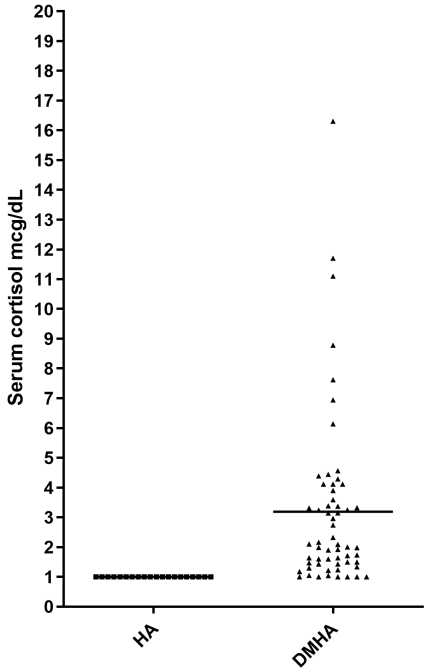

Figure 1: Scatter scale plot comparing basal serum cortisol (BSC) of dogs with hypoadrenocorticism (HA, n = 20) and dogs with disease mimicking hypoadrenocorticism (DMHA, n = 60) The horizontal bars represent the median values.

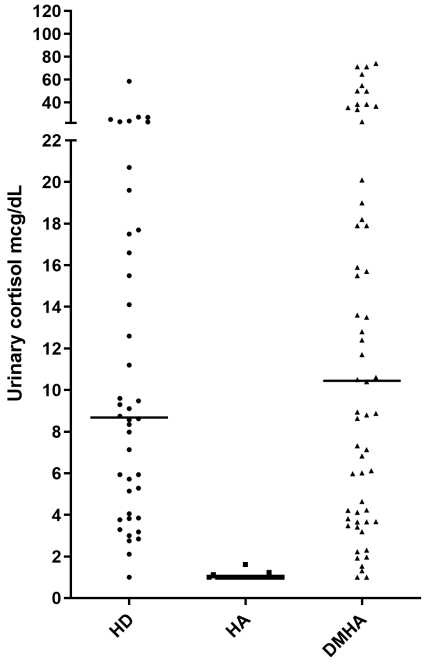

Figure 2: Scatter scale plot comparing urinary cortisol (UC) of healthy dogs (HD, n = 42), dogs with hypoadrenocorticism (HA, n = 20) and dogs with disease mimicking hypoadrenocorticism (DMHA, n = 60) The horizontal bars represent the median values.Two data points in the group DMHA are outside the axis limit.

UCCR

The median UCCR was 9.6 x 10^{-6} (3.9-88.2 x 10^{-6}), 2.5 (1.0-8.2 x 10^{-6}), and 14.7 x 10^{-6} (3.2-401.7 x 10^{-6}) in HD, dogs with HA, and dogs with DMHA, respectively. The UCCR was significantly lower (P < .0001) in dogs with HA as compared to HD and DMHA, but there was no significant difference between HD and DMHA (Figure 3). The area under the ROC curve to discriminate dogs with HA from DMHA was 0.95 (95% CI: 0.90-0.99, Figure 4). A cut-off value of UCCR <8.5 x 10^{-6} revealed 100% sensitivity (95% CI: 83.1-100) and 71.7% specificity (95% CI: 58.6-82.6) in diagnosing HA.

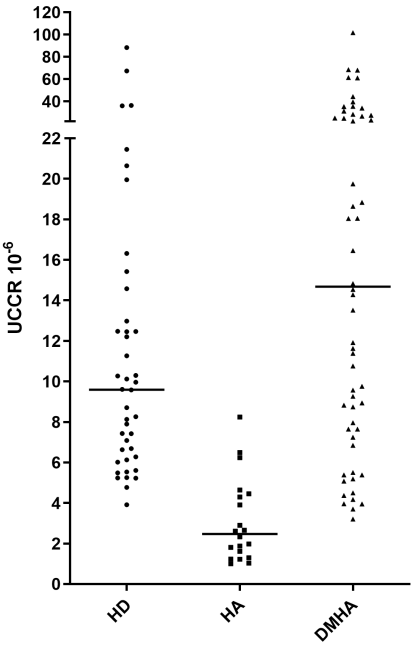

Figure 3: Scatter scale plot comparing urinary cortisol-to-creatinine ratio (UCCR) of healthy dogs (HD, n = 42), dogs with hypoadrenocorticism (HA, n = 20) and dogs with disease mimicking hypoadrenocorticism (DMHA, n = 60) The horizontal bars represent the median values.

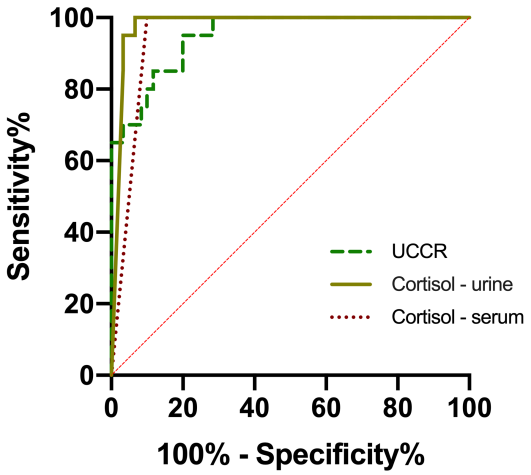

Figure 4: Receiver operating characteristic (ROC) curves assessing basal serum cortisol (BSC), urinary cortisol (UC) and urinary cortisol to creatinine ratio (UCCR) to discriminate dogs with hypoadrenocorticism (HA) from dogs with disease mimicking hypoadrenocorticism (DMHA). The AUC for BSC was 0.95 (95% CI: 0.90-0.99), for UC was 0.98 (95% CI: 0.95-1.00) and for UCCR was 0.95 (95% CI: 0.90-0.99).

DISCUSSION

This study aimed to evaluate the accuracy of UC and UCCR as screening tests for HA in dogs, comparing their performances with the commonly used BSC test. The results indicate that UC and UCCR demonstrate comparable sensitivity but higher specificity than BSC (when using a cut-off value of <2 µg/dL) in identifying dogs with HA. The UC showed the higher accuracy, suggesting its potential as a reliable screening tool.

In this study, the specificity of the BSC in detecting dogs with HA, using the currently recommended cut-off of <2 µg/dL, was 51.1%, a lower value than that found in other studies.[6-12] Such a lower performance of the BSC is presumably related to the fact that the new antibody of CLIA-Immulite for cortisol measurement was used in this study. It has been reported that the new antibody, compared to the previous one, underestimates cortisol not only in urine but also in serum.[15] Such underestimation would explain why many cases of DMHA showed a BSC value <2 µg/dL. However, no dogs with HA showed a BSC >1 µg/dL. Therefore, using the CLIA method with the new antibody, the cut-off value of BSC for ruling out HA should probably no longer be ≥2 µg/dL, but >1 µg/dL.

In a previous study, a cut-off value of UCCR <4.4 x 10^{-6} revealed 100% sensitivity (95% CI: 69.1-100) and 97.3% specificity (95% CI: 85.8-99.9) in detecting HA and in another study UCCR ≤10.0 x 10^{-6} revealed 100% sensitivity (95% CI: 84.6-100) and 100% specificity (95% CI: 95.9-100) in detecting HA.[13,14] In our study, similarly to what was observed with the BSC, the UCCR showed a lower specificity than those reported in the above-mentioned studies. This result can be explained by the use of the new antibody, which, by providing lower cortisol results, causes a higher overlap of the UCCR between dogs with HA and DMHA. Despite the lower diagnostic performances with the new antibody, the UCCR showed a higher specificity than the BSC, when using the cut-off value of BSC <2 μg/dL, and can still be considered a possible screening test for HA in dogs.

UC showed the best performance in discriminating dogs with HA from DMHA. A limitation in considering UC as an absolute value is related to the fact that urine concentration influences the concentration of any urinary analyte. In turn, urine concentration can vary significantly due to factors such as hydration status and renal function. For example, in humans, significantly lower urinary free cortisol values are observed in individuals with moderate-to-severe renal impairment because urinary cortisol excretion is proportional to glomerular filtration rate.[20] This could affect the specificity of UC in dogs with renal impairment screened for HA, resulting in falsely low urinary cortisol concentrations. Moreover, high daily fluid intake can result in higher cortisol excretion rates due to increased urine volumes that reduce the fraction of filtered cortisol metabolized or reabsorbed in the kidney.[21,22] By relating cortisol to creatinine, which is excreted at a relatively constant rate, these variations can be corrected, providing a more accurate reflection of cortisol excretion. Nonetheless, UCCR proved less accurate than UC in the present study. This finding could be related to the limit of detection of the cortisol assay; indeed, urine cortisol concentration below 1 μg/dL was reported as equal to 1 μg/dL, thus falsely increasing the UCCR in dogs with very low urine creatinine concentration and negatively affecting the UCCR specificity to discriminate dogs with HA and dogs with DMHA. The chemiluminescent enzyme immunoassay used in this study to measure serum and urine cortisol is capable of quantifying concentrations of cortisol <1 μg/dL. However, the manufacturer does not guarantee linearity below 1 μg/dL. Therefore, in this study, values <1 μg/dL were considered as 1 μg/dL. However, this study was conducted using dogs with HA and DMHA without selecting them based on urinary concentration; therefore, it reflects the real condition of the clinical setting. Also, urine-specific gravity was not available in all dogs.

Future studies that will also evaluate the influence of urine concentration and renal function may clarify whether the latter may have a significant impact on UC diagnostic performances. However, it is important to underline that UC is a screening test, not a confirmatory test. The suspicion of HA due to a low UC value must then be confirmed with the ACTH stimulation test. Therefore, the evaluation of DMHA dogs with very dilute urine would likely result in more dogs with UC <2 μg/dL; this might lower the specificity of the test but would not cause overdiagnosis of HA.

Another limitation of the present study is that both urine collected at home and at the hospital were used and this potentially affected our results. Previous studies observed significantly increased UCCRs if urine was taken in the hospital compared to at home.[23] Therefore, urine samples for UCCRs measurement in the diagnosis of Cushing's syndrome should be collected in the dog's home environment to avoid the influence of stress on glucocorticoid secretion. The authors of the present study hypothesize that when screening for HA, it would be most appropriate to collect all urine in the hospital, as this would maximize stress and likely allow for further separation of HA and DMHA.

Another limitation of this study is the lack of BSC measurement in healthy dogs, which could provide a baseline for comparison. Additionally, the study relied on stored urine samples, which may introduce variability despite consistent storage conditions.

In conclusion, the study demonstrates that UC and, to a lesser extent, UCCR are promising alternatives to BSC for the initial screening of HA in dogs. The high sensitivity ensures that HA cases are not missed, while the higher specificity compared to BSC performed using the current cut-off (2 µg/dL) means fewer dogs will undergo unnecessary further testing or treatment. Incorporating UC into routine screening tests and applying a lower cut-off for BSC (1 µg/dL) could significantly improve the management of dogs suspected of HA.

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Chapter 5 – Canine Hypercortisolism

5.1 | Re-evaluating Diagnostic Cut-Off Values: Impact of Immulite-2000-Antibody Change on Canine Hypercortisolism Diagnosis Using Low-Dose Dexamethasone Suppression Test

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ABSTRACT

Introduction

The low-dose dexamethasone suppression test (LDDST) is a widely used test for canine hypercortisolism (HC). In 2020, there was a change in the Immulite-2000-antibody used for cortisol measurement and new investigations of LDDST are required.

Objective

The aim of this study was to re-evaluate the LDDST 8-hour cortisol cut-point for the diagnosis of canine HC using a chemiluminescent enzyme immunoassay (Veterinary Cortisol, Siemens, IMMULITE 2000 XPi) and to compare the diagnostic performance of LDDST before and after the change of Immulite-2000-antibody.

Methods

The performance of LDDST was retrospectively evaluated comparing the results in dogs with HC and dogs with disease mimicking HC (DMHC). The diagnosis of HC was based on the presence of compatible clinical signs and at least one of the following criteria: post-ACTH cortisol value >22 µg/dL; undetectable ACTH associated with an adrenal mass; pituitary-to-brain ratio >0.33; clear response to medical treatment. The diagnostic performances of the test were evaluated before (T1, January 2016–October 2020) and after (T2, November 2020–January 2024) the change in the Immulite-2000-antibody. Dogs that received glucocorticoids in the previous 90 days or lacked data for diagnosis confirmation were excluded. The difference between 8-hour cortisol at T1 and T2 in HC dogs was assessed using the Mann-Whitney test. Performance of the LDDST was assessed using sensitivity, specificity and a receiver operating characteristic (ROC) curve.

Results

The LDDST was performed on 63 dogs with HC and 32 dogs with DMHC at T1, and 40 dogs with HC and 40 dogs with DMHC at T2. The median (range) 8-hour cortisol value in HC dogs was 3.35 µg/dL (0.5–25 µg/dL) at T1 and 2.4 µg/dL (0.3–8.9 µg/dL) at T2 (p=0.02). At T1 and T2, the area under the curve for 8-hour cortisol to differentiate HC from DMHC dogs was 0.95 (95% CI 0.91 to 0.99) and 0.97 (95% CI 0.94 to 1.0), respectively. The cut-point associated with the best sensitivity and specificity to diagnose HC was >1.4 µg/dL (Se=88%, 95% CI 77% to 94%; Sp=97%, 95% CI 84% to 100%) at T1 and >1.2 µg/dL (Se=88%, 95% CI 73% to 96%; Sp=98%, 95% CI 87% to 100%) at T2.

Conclusions

This study showed that when using IMMULITE 2000 XPi, the optimal cut-point of LDDST 8-hour cortisol for HC diagnosis was >1.2 µg/dL, which is lower than the currently accepted cut-point of >1.4 µg/dL. Clinicians should consider this updated cut-off value when performing an LDDST for the diagnosis of HC.

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5.2 | Evaluation of Urinary Corticoid-To-Creatinine Ratio Using a Canine-Specific Chemiluminescent Cortisol Assay (Immulite 2000) in the Diagnosis of Canine Hypercortisolism

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ABSTRACT

Introduction

The urinary corticoid-to-creatinine ratio (UCCR) is one of the most commonly used screening tests for canine hypercortisolism (HC). Recently, there was a change in the Immulite- 2000 antibody used for cortisol measurement, and new investigations of UCCR are required.

Objective

The aim of this study was to establish the reference interval (RI) for the UCCR measured with a chemiluminescent enzyme immunoassay (Veterinary Cortisol, Siemens, IMMULITE 2000 XPi) and to investigate the diagnostic performance of this method for UCCR in canine HC.

Methods

The UCCR was determined on urine samples from healthy dogs (HD), dogs with HC and dogs with diseases mimicking HC (DMHC). The HD group included dogs with no clinical signs and normal blood tests and urinalysis. Dogs were included in the HC group if they had compatible clinical signs and clinicopathological abnormalities coupled with a positive ACTH stimulation test and/or low-dose dexamethasone suppression test. The DMHC group included dogs for which HC was suspected based on clinical signs and/or ultrasonographic evidence of an adrenal mass but was subsequently excluded. Dogs were excluded from the study if glucocorticoids were administered in the previous 90 days before testing.

Results

The study included 40 HD, 97 dogs with HC and 35 dogs with DMHC. In all HD, urine was collected by free catch at home (AH) and in 26 dogs HD also in the hospital (IH). The RI for UCCR, established on urine collected AH, was between 3×10-6 (90% CI 2.3–3.8) and 26×10-6 (90% CI 29.7–35.0). The median (min-max) UCCR results were significantly higher for IH samples (11.7×10-6; 5.3–45.8×10-6) compared to those collected AH (8.19×10-6; 3.9–36.3×10-6; p=0.03). UCCR in dogs with HC (70.9×10-6;6.8–882.2×10- 6) was significantly higher than that HD (9.1×10-6, 3.9–36.3×10-6; p<0.001) and dogs with DMHC (15×10-6, 2.63–137.8×10-6; p<0.001). The area under the ROC curve for UCCR to differentiate HC dogs from dogs with DMHC was 0.85 (95% CI 0.78–0.92). Using as a cut-off value the upper limit of the RI (UCCR>26×10-6), the sensitivity and the specificity for the UCCR in detecting HC were 80.4% (95% CI 71.1–87.8) and 71.4% (95% CI 53.7–85.4), respectively.

Conclusions

This study established a new RI for UCCR using IMMULITE 2000 XPi in dogs, and we confirmed the importance of collecting urine AH to avoid the influence of stress on UCCR results. Using the upper limit of the RI, the sensitivity of this test for diagnosis of HC resulted lower than previously reported. Therefore, UCCR should not be used alone to exclude HC in dogs.

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5.3 | Effects of Dietary Intervention on Calcium and Phosphate Homeostasis in Dogs with Naturally Occurring Hypercortisolism

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Abstract

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ABSTRACT

Introduction

Naturally occurring hypercortisolism (HC) affects calcium and phosphate homeostasis through multiple mechanisms. This condition, previously referred to as "adrenal secondary hyperparathyroidism", may resolve in dogs undergoing medical treatment with trilostane. However, the combination of medical and nutritional treatment on calcium-phosphate homeostasis in dogs with HC has not yet been investigated.

Objective

This prospective study aimed to evaluate the effects of a therapeutic commercial diet (TCD), formulated for the management of canine calcium oxalate (CaOx) urolithiasis, on calcium and phosphate homeostasis in dogs with HC treated with trilostane. This diet was selected because both CaOx urolithiasis and HC are characterized by increased urinary calcium excretion.

Methods

Dogs diagnosed with HC (pituitary or adrenal-dependent) treated with trilostane for at least one month were prospectively enrolled (2021-2023). Age and body weight matched-healthy dogs were included as a control group (CG). Dogs receiving medications or TCDs known to affect calcium and phosphate homeostasis (e.g., renal diets), and those with severe comorbidities (e.g., diabetes mellitus, other neoplasia), were excluded from the study. Dogs with HC were fed a dry TCD (on a dry matter basis: Ca=0.6%, P=0.4%) for a 2-month period, while CG dogs continued their diet. Ionized calcium (iCa), chemistry profile, urinalysis including urinary fractional excretion (%) of phosphate (FEP) and calcium (FECa), serum PTH (sPTH), 25-hydroxyvitamin D (25D), calcitriol (1,25D), fibroblast growth factor-23 (FGF-23) concentrations were evaluated in all dogs at inclusion (T0) and only in dogs with HC after 2 months of dietary treatment (T2). Data were reported as median and range, and analyzed using nonparametric statistics.

Results

Fifteen HC and 15 healthy dogs were included. At T0, dogs with HC had higher FECa and sPTH concentration (Figure 1A) compared with CG (0.31 [0.09-0.93] vs 0.15 [0.06-0.43] %, p=0.01; and 6.9 [2.3–13.8] vs 3.1[0.6–8.4] pmol/L, p=0.0003, respectively). Serum phosphate, iCa concentrations, and FEP did not differ between groups. Serum 1,25D and FGF-23 concentrations were lower in HC compared with CG (321 [232–394] vs 398 [230–507] pmol/L, p=0.003; and 312 [171–508] vs 424 [235–753] pg/mL, p=0.03, respectively; Figure 1B-C), while serum 25D concentration did not differ between groups (Figure 1D). In dogs with HC, sPTH and 1,25D concentrations decreased (median difference -2.3 [95% CI -3.7 to -0.3] pmol/L, p=0.02; and -38 [-64 to -11] pmol/L, p=0.01, respectively; Figure 1A-B), while 25D concentration increased at T2 (median difference 52 [95% CI 11 to 73] nmol/L, p=0.02; Figure 1D). FGF-23 tended to increase at T2, but the difference was not significant (median difference 29 [95% CI -3 to 63] pg/mL, p=0.06; Figure 1C). Serum phosphate, iCa concentrations, FECa and FEP showed no significant differences between T0 and T2. Trilostane dosage did not differ between T0 and T2 (median 1.0 [range, 0.3-2.4] vs 1.2 [0.3-2.4] mg/kg, p=0.2, respectively).

Conclusions

The use of a CaOx TCD may help restore calcium and phosphate homeostasis in dogs with HC undergoing treatment with trilostane. The primary limitation of this study lies in the administration of trilostane itself, which may have affected calcium and phosphate homeostasis.

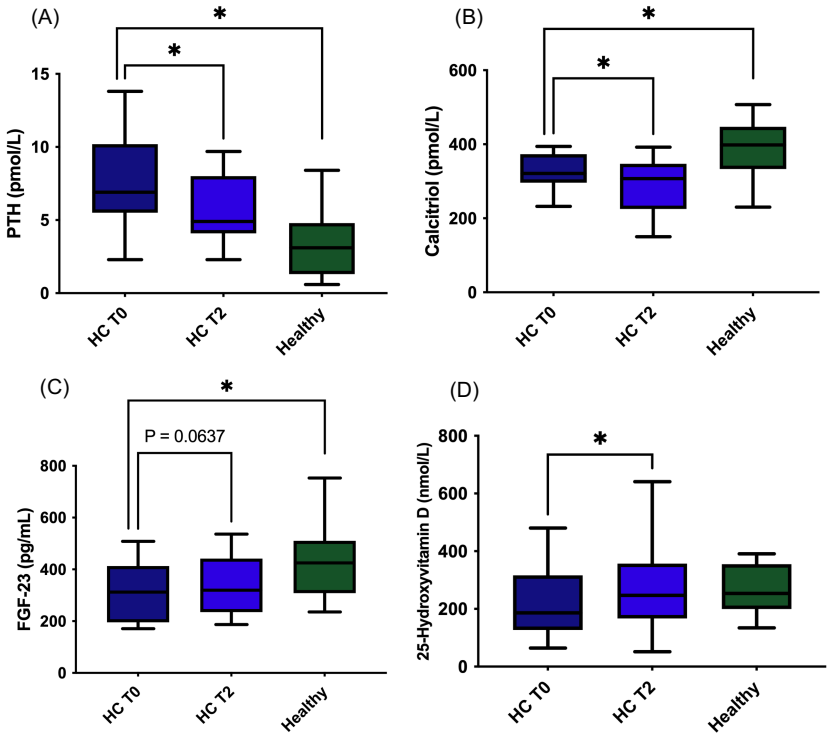

Figure 1. Box and whiskers plots comparing concentrations of (A) parathyroid hormone (PTH), (B) Calcitriol, (C) fibroblast growth factor-23 (FGF-23), and (D) 25-hydroxyvitamin D in dogs with naturally occurring hypercortisolism (HC, T0 and T2) and healthy dogs. The boxes represent the interquartile range from the 25th to the 75th percentile. The horizontal bar in each box represents the median value. The whiskers represent the range (Min-Max value). * $P < 0.05$

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Chapter 6

Summarizing discussion and conclusions

This thesis presents advancements in diagnostic, therapeutic, and monitoring approaches for canine and feline endocrinopathies. **Chapter 3** discusses the application of novel insulin analogs in diabetic dogs and examines various aspects of continuous glucose monitoring systems (CGMS) in diabetic veterinary patients. **Chapters 4** and **5** present research studies that evaluate diagnostic approaches for naturally occurring hypoadrenocorticism (HA) and hypercortisolism (HC) in dogs, with a focus on misdiagnosis risk and the performance of adrenal function tests using the updated cortisol assay.

Diabetes mellitus (DM) is a common endocrine disorder in dogs. Nutritional management plays a crucial role in the long-term management of diabetic dogs.[1] However, no consensus exists regarding the ideal composition and macronutrient balance in dietary formulations for DM in dogs.[1,2] The nutritional management of diabetic dogs has traditionally relied on commercially diets (CDs), while alternative nutritional strategies, such as homemade diets (HMDs), have not been previously investigated in research studies. The latter could prove beneficial for diabetic dogs, as their nutritional content can be customized to meet the individual patient's needs. **Chapter 3.1** presents a randomized crossover study that evaluates the effects of a therapeutic veterinary diabetic dry CD and a HMD on glycemic control and glycemic variability in ten client-owned dogs with stabilized DM, monitored using a CGMS. The results indicated that diabetic dogs fed the HMD had similar body weight, body condition score, exogenous insulin requirements, and glycemic control levels compared to those fed the CD. However, when evaluating CGMS-derived metrics, the HMD was associated with a significant reduction in the interstitial glucose (IG) percentage of time above range (TAR%) and an increase in the percentage of time below range (TBR%) at specific time points. Additionally, dogs fed the HMD showed reduced serum cholesterol concentrations compared to those fed the CD. These findings may be attributed to differences in the ingredients and cooking processes of the two diets. In contrast, percentage of time in range (TIR%) and glycemic variability metrics did not show significant differences when diabetic dogs were fed either the CD or the HMD. In conclusion, both the CD and the HMD can be considered valid dietary options for managing DM in dogs. These results suggest that the HMD formulated for this study may have a more effective glucose-lowering effect compared to the CD.

The management of DM in dogs aims to resolve or improve clinical signs, minimize potential complications, and ensure a high quality of life (QoL) for both the dog and the owner.[3] Given the high risk of euthanasia for diabetic dogs if the owner feels unable to cope with the requirements of treatment, maintenance of the companion animal-human bond should be prioritized when discussing therapeutic options.[4] The main concerns reported by owners of diabetic dogs relate to the impact of the daily treatment schedule on their quality of life.[5] Providing practical alternatives, such as the use of “basal” insulin, may allow for more flexible feeding schedules and once-daily dosing, thereby significantly easing the caregiving burden for many owners. **Chapter 3.2** and **3.3** describe two treatment protocol using insulin glargine 300 U/mL (IGla300) or insulin degludec 100 U/mL (IDeg) as basal once-daily insulin in client-owned diabetic dogs with FreeStyle Libre CGMS used for dose titration. In a prospective evaluation, IGla300 was administered to 95 client-owned diabetic dogs and achieved good to excellent glycemic control in the majority (92%), including those with concurrent diseases. IGla300 was administered once daily in 59% of dogs with concurrent diseases and in 72% of dogs without. The median dose required to achieve glycemic control was 1.9 U/kg/day, with a range up to 5.2 U/kg/day, which is notably higher than doses typically needed with other insulin formulations. The use of IDeg was prospectively assessed in 33 client-owned diabetic dogs, with 76% achieving excellent or very good diabetic control. Eighty five percent of dogs were maintained on once-daily insulin dosing throughout the study, and the final insulin dose was 1.3 U/kg/day (range 0.4–2.2 U/kg/day). Both IGla300 and IDeg were associated with a low incidence of clinical hypoglycemia (6% and 3%, respectively). In both studies, continuous glucose monitoring facilitated clinical and glycemic improvement within a relatively short time period. In conclusion, basal insulin treatment of diabetic dogs with IGla300 and IDeg provides a

practical alternative to traditional treatment approaches using q12h injections of intermediate-acting insulin formulations and regular feeding of meals. This novel protocol represents a paradigm shift in the overall strategy of DM treatment in dogs, because it uncouples insulin injections from feeding, providing owners with more flexibility in terms of timing, type, and consistency of meals. It thus provides an opportunity to improve the QoL and alleviate the treatment burden for many caregivers of diabetic dogs. Clinicians should be aware that, in some dogs, twice-daily administration with or without meal-time bolus injections may be necessary to achieve glycemic control. Moreover, monitoring with CGMS is essential for dose titration of basal insulin.

Hypoglycemia is a primary limiting factor in managing DM in patients receiving insulin therapy, and fear of hypoglycemia is one of the most significant factors negatively impacting the QoL for owners of diabetic pets.[5,6] Transmucosal glucagon formulations, currently used in human diabetic care, could potentially be administered at home by pet owners to treat life-threatening hypoglycemia without requiring technical expertise. Their use may also improve the QoL for diabetic pet owners by reducing their fear of hypoglycemia. **Chapter 3.4** shows that Baqsimi, an intranasal glucagon powder recently approved for use in diabetic people,[7] rapidly increased blood glucose (BG) concentrations when administered intranasally and rectally in six healthy cats. Rectal administration of Baqsimi was associated with greater increases in BG and glucagon concentrations; however, it remains unclear whether this difference was due to inconsistent administration in the nasal group or reduced absorption. The administration of the drug was well-tolerated, with only mild and transient adverse effects such as sneezing, hypersalivation, blepharospasm, and vomiting. Future studies are needed to further evaluate the efficacy and safety of transmucosal glucagon in diabetic cats, particularly during hypoglycemic events.

The FreeStyle Libre is currently the most studied CGMS in veterinary patients.[8] In diabetic people, FSL3 offers increased accuracy,[9] and its smaller size could be advantageous for use in veterinary patients. **Chapter 3.5** shows that FSL3 provides clinically accurate measurements in the euglycemic and hyperglycemic ranges in diabetic cats. Similar to previous veterinary studies utilizing the ISO 2013 guidelines, the FSL3 did not meet the standards for analytical accuracy. However, the mean absolute relative difference (MARD) value was lower (13.4%) than those reported in veterinary studies evaluating the accuracy of previous FSL models.[10,11] The shortest sensor lifespan with FSL3 (4 days) was longer than that reported in earlier studies in diabetic cats (1–2 days), suggesting improved tolerability of the FSL3 potentially due to its smaller size. One important limitation of this study was the low number of data points in the hypoglycemic range, which is crucial for clinical decision-making. Thus, the aim of the study presented in **Chapter 3.6** was to assess the accuracy of FSL3 in cats with experimentally-induced hypoglycemia. The results showed a good clinical accuracy of FSL3 during hypo- and euglycemia, but ISO 2013 standards for analytical accuracy were not met. Interstitial glucose concentration measured by FSL3 underestimated BG in euglycemia and mild hypoglycemia, with the difference decreasing as BG levels decrease. However, in cases of marked hypoglycemia (<55 mg/dL), IG overestimated BG in healthy cats. In conclusion, the FSL3 provides clinically accurate measurements in the hyperglycemic, euglycemic and hypoglycemic ranges. Clinical interventions prompted by IG measurements within the hypoglycemic range can have significant consequences. Therefore, recognizing the proportional glycemic-dependent bias associated with FSL3 IG enable clinicians to enhance the safety of its application in feline patients.

The Libreview system generates comprehensive glucose reports from the IG data of the FreeStyle Libre, including the Ambulatory Glucose Profile (AGP). The AGP report provides both a visual and a statistical summary of the glucose metrics such as mean glucose (MG), TIR%, TAR%, and TBR%, along with glycemic variability expressed as percent coefficient of variation (CV%).[8] Although the FreeStyle Libre is increasingly used in diabetic dogs, its integration

into routine clinical practice remains limited due to the absence of standardized guidelines for data interpretation. **Chapter 3.7** presents findings from a preliminary study evaluating the utility of various metrics provided by the AGP report of the Libreview system for monitoring glycemic control in diabetic dogs. Metrics such as TIR%, TAR%, TBR%, and MG showed moderate/strong correlations with the clinical score, underscoring their relevance in guiding insulin management. Furthermore, the coefficient of variation (CV%) was higher in dogs with concurrent diseases and in those experiencing clinical hypoglycemia, suggesting a potential association between increased glucose variability and these conditions in diabetic dogs. These results suggest that FreeStyle Libre-derived metrics, hold potential for assessing glycemic control in diabetic dogs. These metrics offer valuable insights into glucose trends, complementing traditional clinical assessments and facilitating more precise insulin management. Further research is warranted to establish standardized guidelines for their use in veterinary practice and to explore their role in improving long-term outcomes in canine diabetes management.

In veterinary medicine, it is generally accepted that owner compliance is essential for successfully treating DM.[4] The disease and the treatment commitments are likely to have a considerable impact on owners' daily routines and QoL and might represent a significant temporal, financial, and emotional burden. Therefore, it is important to consider the impact of DM management and of the different monitoring methods on the QoL of pet owners. **Chapter 3.8** shows the results of a retrospective study aimed to evaluate the impact of FreeStyle Libre on the quality of life of diabetic pet owners. Fifty diabetic pet owners who used at least one FreeStyle Libre on their diabetic pet were asked to answer a 30-question survey. A total of 92% of diabetic pet owners reported that their pet had better diabetes control since using the device; while the most challenging aspects were ensuring proper sensor fixation during the wearing period (47%), preventing premature detachment (40%), and purchasing the sensor (34%). Moreover, 36% of owners reported that the device cost was difficult to afford in the long term. Comparing dogs and cats, a significantly higher number of dogs' owners found the device to be well-tolerated (79% vs. 40%), less invasive than blood glucose curves (79% vs. 43%), and easier to maintain in situ (76% vs. 43%). In conclusion, the FreeStyle Libre is considered by diabetic pet owners to be easy to use and less stressful compared to blood glucose curves, while also enabling better glycemic control. Nevertheless, costs related to its long-term use might be difficult to sustain. The main limitation of the study was that all diabetic patients included were monitored at a referral center, which may have positively influenced the results. Therefore, additional studies that also include diabetic pets managed by primary care veterinarians are warranted.

The Eversense XL continuous glucose monitoring system has recently been developed for monitoring DM in humans. The sensor is fully implanted and has a lifespan of up to 180 days.[12] **Chapter 3.9** presents a case series that describes, for the first time, the clinical use of this novel CGMS in three diabetic dogs. The insertion and use of the device were straightforward and well tolerated by the dogs. During the wearing period, some device-related drawbacks were reported, including sensor dislocation and the need for daily calibrations. A good correlation was observed between the glucose values measured by the Eversense XL and those obtained with FreeStyle Libre and a portable blood glucose meter, previously validated for use in diabetic dogs ($r_s = 0.85$ and $r_s = 0.81$, respectively). The sensor lifespan was 180 days in two of the three dogs, and the use of the device provided high satisfaction to the owners. In conclusion, this novel long-term implantable CGMS appeared to be well tolerated and strongly correlated with two commercially available devices previously validated for use in DD. In general, the Eversense XL might be considered a future alternative for glucose monitoring and could positively impact the adherence and long-term use of CGMSs in diabetic dogs. However, the use of this device in veterinary medicine could have some limitations, such as excessive movement of the sensor, the need for daily calibrations, high costs, and limited availability. Further investigations are needed to determine the accuracy of this CGMS in diabetic dogs.

Hypoadrenocorticism is a rare endocrinopathy in dogs.[13] According to the Agreeing Language in Veterinary Endocrinology (ALIVE) project of the European Society of Veterinary Endocrinology (ESVE), the term 'eunatraemic, eukalaemic hypoadrenocorticism' (EEH) is used to describe the form of the disease characterized by normal serum electrolyte concentrations.[14] Eunatremic, eukalemic hypoadrenocorticism might be mistaken for other diseases, such as chronic gastrointestinal disease (CGD), due to the vague clinical signs and the absence of typical biochemical abnormalities. Additionally, previous administration of glucocorticoids, frequently used in dogs with CGD, can lead to false positive results on the adrenal function tests, potentially resulting in a misdiagnosis of EEH. **Chapter 4.1** shows the results of a study aimed to determine the prevalence of EEH in dogs with signs of CGD, and to identify clinical and clinicopathological features for EEH and previous glucocorticoid administration. In this multicenter prospective study, the prevalence of EEH in a cohort of 112 dogs with CGD presented to 2 referral institutions was 0.9% (95% CI, 0.1%-4.8%), demonstrating a lower prevalence of EEH than that reported in previous studies.[15,16] The basal serum cortisol concentration was <2 µg/dL in 9 of 11 (82%) dogs that had received glucocorticoids and in 48 of 101 (47.5%) dogs that had not. Additionally, the ACTH stimulation test (ACTHst) provided false-positive results in 2/11 dogs previously treated with glucocorticoids. Currently, no guidelines exist regarding the required time until the ACTHst can be carried out after a dog has been treated with different glucocorticoid formulations. In dogs, duration of hypothalamic-pituitary-adrenal (HPA) axis suppression after systemic glucocorticoid treatment is reported to vary from a few days to up to 7 weeks after glucocorticoid discontinuation.[17-23] **Chapter 4.2** presents a single-center prospective observational study aimed at determining the timeline for recovery of the hypothalamic-pituitary-adrenal (HPA) axis in a group of 20 ill dogs treated with intermediate-acting glucocorticoids. The results indicated that, in dogs treated with systemic intermediate-acting glucocorticoids for at least 7 days, the median time for HPA axis recovery was 3 days. Approximately half of the dogs (11 of 20) experienced complete recovery of the HPA axis within a few days following glucocorticoid discontinuation. However, 2 of 20 dogs required more than 8 weeks to achieve complete HPA axis recovery. The glucocorticoid dose and duration of treatment were not correlated with the timing of HPA axis recovery. Additionally, the timing of HPA axis recovery in dogs undergoing an alternate-day tapering dose was not significantly different compared to dogs that did not follow this approach (3.5 days vs. 3 days). In conclusion, the results of these studies indicate that the prevalence of EEH is less than 1% in dogs presenting with signs of CGD, underscoring the critical importance of excluding previous glucocorticoid administration to prevent misdiagnosis of EEH. The optimal time to test for HPA axis recovery after GC use remains controversial because the variability of data regarding the recovery timelines. Clinicians should be aware that, after prolonged treatment with intermediate-acting glucocorticoids, HPA axis recovery can occur as early as 2 to 6 days after discontinuation, although some dogs may require more than 8 weeks. Given that HA requires lifelong treatment, measuring endogenous ACTH and repeating the ACTH stimulation test is recommended in dogs with an unclear history of glucocorticoid exposure.

Measurement of a resting (basal) cortisol concentration, a simple and cost-effective screening test, is commonly used to rule out hypoadrenocorticism.[24-27] However, due to the low specificity of this test, urinary corticoid-to-creatinine ratio (UCCR) has been proposed as alternative screening test for HA in dogs.[28] In the study presented in **Chapter 4.3,** a UCCR cut-off value of $<4.4 \times 10^{-6}$ yielded 100% sensitivity and 97.3% specificity in diagnosing HA. In this study, urinary cortisol was measured using a chemiluminescent immunoassay (CLIA) (Immulite 2000 cortisol). After analyzing the samples of the above-mentioned study, there was a change in the Immulite 2000 antibody used for cortisol measurement. An initial review by the Endocrine Quality Assurance program of the ESVE, based on more than 40 canine urine samples, indicated that cortisol values measured with the new antibody were on average 70% lower than those obtained with the previous antibody.[29] These findings suggest that the new antibody may alter diagnostic performance. Therefore, the study

presented in **Chapter 4.4** aimed to establish new reference intervals and evaluate the diagnostic performance of urinary cortisol and UCCR for diagnosing HA using the currently available cortisol antibody. The study results demonstrated that urinary cortisol and UCCR had comparable sensitivity but higher specificity than basal serum cortisol (when using at a cut-off value <2 µg/dL) for identifying dogs with HA. A UCCR cut-off value of $<8.5 \times 10^{-6}$ yielded 100% sensitivity and 72% specificity for diagnosing HA. Urinary cortisol showed the highest accuracy, with a cut-off of <2 µg/dL achieving 100% sensitivity and 90% specificity. These findings suggest that urinary cortisol and UCCR are effective alternatives to basal serum cortisol for the initial screening of HA in dogs, providing high sensitivity to minimize missed HA diagnoses and greater specificity to reduce unnecessary additional testing or treatments. A main limitation of this study is the lack of standardization in urine collection, with samples collected both at home and in the hospital, which may have influenced the results.

Naturally occurring HC, also known as Cushing's syndrome, is a common endocrinopathy in dogs.[30-32] Diagnosing HC is a complex process that necessitates a comprehensive assessment of clinical signs, clinicopathological abnormalities, imaging findings, and endocrine test results.[33] The diagnostic performance of adrenal function tests used for HC diagnosis has been previously evaluated.[33,34] However, a recent change in the Immulite 2000 antibody used for cortisol measurement has introduced an average bias of -23% in canine serum and -70% in urine.[29] As a result, previously established cut-off points for diagnosing canine HC require re-evaluation. **Chapters 5.1** and **5.2** focus on the results of two abstracts aimed to evaluate new reference intervals and assess the diagnostic performance of the low-dose dexamethasone suppression test (LDDST) and UCCR using the new cortisol antibody. In **Chapter 5.1**, the diagnostic performance of the LDDST were evaluated before (January 2016–October 2020) and after (November 2020–January 2024) the change in the Immulite-2000-antibody. The median 8-hour cortisol value in HC dogs was significantly different before and after the change of Immulite-2000-antibody (3.35 µg/dL vs. 2.4 µg/dL, respectively). The cut-off associated with the best sensitivity and specificity to diagnose HC was >1.4 µg/dL (88% sensitivity, 97% specificity) before and >1.2 µg/dL (88% sensitivity and 98% specificity) after the antibody change. This study showed that when using IMMULITE 2000 XPi, the optimal cut-point of LDDST 8-hour cortisol for HC diagnosis was >1.2 µg/dL, which is lower than the currently accepted cut-point of >1.4 µg/dL. Clinicians should consider this updated cut-off value when performing an LDDST for the diagnosis of HC. In **Chapter 5.2**, the reference interval for UCCR, based on urine collected at home, ranged from 3×10^{-6} to 26×10^{-6}. Median UCCR values were significantly higher for samples collected in the hospital compared to those collected at home. When using the upper limit of the reference interval (UCCR $>26\times10^{-6}$) as a cut-off, the sensitivity and specificity of UCCR for detecting HC were 80.4% and 71.4%, respectively. In conclusion, this study established a new UCCR reference interval using the IMMULITE 2000 XPi in dogs and highlighted the importance of home urine collection to minimize stress-related impacts on UCCR results. Notably, the sensitivity of UCCR for diagnosing HC, using this upper reference limit, was lower than previously reported.[35] Consequently, UCCR should not be used as a sole test to exclude HC in dogs.

Cushing's syndrome can affect calcium and phosphate homeostasis through multiple mechanisms. In dogs with HC, disruptions in calcium-phosphate hemeostasis are often characterized by hyperphosphatemia, elevated serum parathyroid hormone (PTH) concentrations, decreased urinary phosphate excretion, and increased urinary calcium excretion.[36-38] Additionally, a recent study reported lower serum 25-(OH)-Vitamin D and plasma fibroblast growth factor-23 (FGF-23) concentrations in dogs with HC compared to controls.[38] This condition, previously referred to as "adrenal secondary hyperparathyroidism", may resolve in dogs undergoing medical treatment with trilostane.[37] Chapter 5.3 presents preliminary results from a study evaluating the effects of a therapeutic commercial diet (TCD), formulated for the management of canine calcium oxalate (CaOx) urolithiasis, on calcium and phosphate homeostasis in 15 dogs with HC

treated with trilostane. After feeding the TCD for a 2 month-period, dogs with HC showed significantly decreased serum PTH and calcitriol concentrations (median differences of -2.3 pmol/L and -38 pmol/L, respectively), while serum 25-(OH)-Vitamin D concentrations significantly increased (median difference 52 nmol/L). Additionally, plasma FGF-23 concentration showed an upward trend (median difference 29 pg/mL), although this increase was not statistically significant (p=0.06). These preliminary findings suggest that a TCD formulated for the management of canine CaOx urolithiasis may aid in restoring calcium and phosphate homeostasis in dogs with HC undergoing trilostane therapy. Although the trilostane dosage remained stable throughout the study, a primary limitation is the potential impact of trilostane itself on calcium and phosphate homeostasis. Further studies are needed to determine whether long-term use of a TCD marketed for CaOx urolithiasis could provide sustained benefits in managing dogs with HC.

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