Master's Thesis

Saliva glucose test in canine diabetes monitoring

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Because incidence of diabetes mellitus in dog has increased, easy and efficient glucose monitoring techniques are needed. Saliva sampling offers a straightforward and non-invasive method for measuring glucose levels in dogs, providing a unique way to study disease markers. The low α-amylase levels in canine saliva ensure stable glucose measurements without the need for invasive procedures. This thesis aims to evaluate the effectiveness of the prototype 2 saliva glucose test for monitoring glucose levels in dogs. We compared the saliva test results to simultaneous traditional blood and urine glucose tests and to continue monitoring of interstitial glucose level with a skin-attached electronic device. Additionally, the owners of the dogs gave feedback on the tests' usability. The Prototype 2 saliva glucose test showed a 71.3% accuracy in a study of 164 samples, with 80.5% sensitivity and 66.6% specificity. Moreover, the saliva test demonstrated moderate discriminative ability, with an Area Under Curve (AUC) value of 0.708. The dogs' owners feedback revealed high satisfaction with the tests' non-invasive nature and easy use. The Prototype 2 saliva glucose test has the potential to significantly improve canine diabetes management by offering a less stressful and more accessible monitoring option for pet owners. But this test still needs further development.

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Koska diabeteksen esiintyvyys koirilla on kasvanut, tarvitaan helppoja ja tehokkaita glukoosin seurantamenetelmiä. Sylkinäytteiden ottaminen tarjoaa suoran ja ei-invasiivisen menetelmän glukoosipitoisuuksien mittaamiseen koirilla, mahdollistaen taudin merkkiaineiden tutkimisen. Koiran syljen matalat α-amylaasipitoisuudet takaavat stabiilit glukoosimittaukset ilman invasiivisia toimenpiteitä. Tässä tutkielmassa arvioitiin prototyypin 2 sylkiglukoositestin tehokkuutta koirien glukoosipitoisuuksien seurannassa. Vertasimme sylkitestin tuloksia samanaikaisiin perinteisiin veren ja virtsan glukoositesteihin sekä jatkuvaa solunesteen glukoosipitoisuuden seurantaa ihoon kiinnitetyllä elektronisella laitteella. Lisäksi koirien omistajilta kerättiin palautetta testin käytettävyydestä. Prototyypin 2 sylkiglukoositesti osoitti 71,3 % tarkkuuden tutkimuksessa, jossa otettiin 164 testiä. Testillä oli 80,5 % herkkyys ja 66,6 % spesifisyys 10 koiran otoksessa (viisi diabeetikkoa ja viisi tervettä koiraa). Vaikka testin diskriminointikyky oli kohtalainen (AUC 0,708), koirien omistajien antama palaute korostaa sen potentiaalia helpottaa koirien diabeteksen hallintaa. On kuitenkin selvää, että testi tarvitsee vielä lisäkehitystä.

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TERMS AND ABBREVIATIONS

TERMS

α-amylase	Enzyme that breaks down carbohydrates
Autoimmune response	Immune system attacks body's own cells
Biomarkers	Measurable indicators of a biological state
Glucagon	Hormone that increases blood glucose levels
Glucogenolysis	Breakdown of glycogen to release glucose
Gluconeogenesis	Production of glucose from non-carbohydrate sources
Glucosuria	Excess glucose in the urine
Glycogenesis	Formation of glycogen from glucose
Hyperadrenocorticism	Excessive adrenal gland activity
Hypothyroidism	Underactive thyroid gland
Invasive	Requires entry into the body, often through a procedure
Non-invasive	Does not require entry into the body, typically external
Postabsorptive state	Body in a fasting state
Postprandial state	Body in a fed state after eating

ABBREVIATIONS

AUC	Area under the curve
BCS	Body condition score
NPV	Negative predictive value
PPV	Positive predictive value

1 INTRODUCTION

Diabetes mellitus is a prevalent chronic endocrine disorder affecting dogs globally, with a mainly increasing incidence particularly in the United States (O'Kell and Davison 2023). The prevalence of diabetes in pet dogs is estimated to be between 0.26% and 0.36% of the total dog population (Heeley et al. 2020, Denyer et al. 2021). This rise is likely due to changes in dogs' lifestyles influenced by human urbanization trends and a preference for breeds predisposed to diabetes mellitus (Kumar et al. 2014, Álvarez-Linares et al. 2017, Denyer et al. 2021, O'Kell and Davison 2023). Examples of breeds with higher proclivity to develop diabetes mellitus include Samoyeds, Tibetan terriers, Cairn terriers, and Yorkshire terriers, while breeds like German shepherds, Golden retrievers, and boxers demonstrate a lower incidence (Heeley et al. 2020).

Diabetes in dogs can arise at any age, but most diabetic dogs are middle-aged to geriatric. Female dogs are affected twice as often as male dogs, and obesity is a significant risk factor for the development of the disease. Common signs of diabetes mellitus in dogs include glucosuria (excess glucose in urine), increased drinking, and increased urination (Bagchi and Nair 2012, Feldman et al. 2015, André et al. 2017).

Diabetes mellitus in dogs is a complex and multifaceted disease that is influenced by a variety of factors, including genetics, immune-related issues, pancreatitis, obesity, drugs, infections, and hyperlipemia (Moshref et al. 2019, Heeley et al. 2020). The disease in dogs is characterized by an absolute deficiency of insulin due to the destruction of pancreatic β-cells or by insulin resistance, where the body's response to insulin is diminished, leading to the body's inability to regulate blood sugar levels effectively (Saltiel and Kahn 2001, Feldman et al. 2015, Grau 2023). Genetic predispositions, particularly major histocompatibility complex class II genes and dog leukocyte antigen, play a significant role in the development of diabetes mellitus by contributing to autoimmune responses that can lead to the destruction of pancreatic β-cells (Heeley et al. 2020, Grau 2023).

Diabetic dogs often exhibit histological changes in their pancreases, such as reduced islets and beta-cell issues, which are critical for insulin production. Additionally, structural changes within the pancreas, such as vacuolation of islet cells and ductal epithelium, further elucidate the pathophysiological changes caused by diabetes mellitus (Grau 2023). Diabetic dogs often suffer from concurrent disorders, such as hyperadrenocorticism, urinary tract infections, dermatitis, otitis, and hypothyroidism, which can complicate the clinical picture and influence disease progression and management (Heeley et al. 2020; Yoon et al. 2020). Hyperadrenocorticism has been identified as the most common endocrine disorder associated with diabetes mellitus. The condition leading to excessive cortisol production is known to counteract insulin's effects. This increases the risk of developing diabetes (Heeley et al. 2020).

The role of the immune system, particularly immune-mediated insulitis, and exocrine pancreatic disease, such as pancreatitis, has also been implicated in the development of diabetes mellitus (Nelson 2015). The presence of pancreatitis has been frequently observed in diabetic dogs, suggesting a link that may be both contributory and concurrent to diabetes mellitus (Heeley et al. 2020). Pancreatitis has been associated with an increased risk of developing diabetes mellitus and decreased survival rates in affected dogs (Saltiel and Kahn 2001, Feldman et al. 2015, Grau 2023).

Currently, the diagnosis of diabetes mellitus in dogs mainly relies on blood tests to measure glucose levels and evaluate insulin function, which is invasive and can be stressful for the animals (König et al. 2012, Del Baldo et al. 2020, Grau 2023). These procedures typically require veterinary clinic visits, which are time-consuming and could delay the initiation of necessary treatments. Furthermore, diabetes mellitus often goes undiagnosed due to subtle early symptoms and the limitations of existing test methods. Therefore, considering the complexity of diabetes mellitus and the risk of rapid health decline in dogs, there is a critical need for more streamlined diagnostic methods.

Early and precise diagnosis is vital for beginning timely treatment, which can significantly improve diabetic dogs' quality of life and increase their survival rates. This thesis researches an innovative saliva glucose test prototype 2 that aims to address the challenges of early detection and management of diabetes mellitus in dogs. This saliva test aims to offer rapid diagnosis, thereby preventing premature deaths and improving disease management.

1.1 Dogs' saliva role and compounds

Saliva is a sophisticated and versatile fluid that plays a crucial role in maintaining both the soft and hard tissues of the mouth. Saliva contains a wealth of biological markers- including hormones, enzymes, immunoglobulins, and micro-RNA molecules- that reflect the body's health status, making it valuable resource for medical diagnostics. The analysis of these biomarkers can reveal information about various physiological and pathological conditions, including metabolic disorders, infections, cancers, and even neurodegenerative diseases (Muñoz-Prieto et al. 2019).

In dogs, key saliva producers are the parotid ducts from the parotid gland, the zygomatic duct from the zygomatic gland, and the mandibular duct from the mandibular gland. These ducts are instrumental in delivering saliva to the oral cavity. Saliva has many different functions, such as food digestion, protecting against microbial invasion and helping cleanse the oral mucosa and teeth from harmful substances (Pasha et al. 2018, Sanguansermsri et al. 2018, Muñoz-Prieto et al. 2019).

Dogs have different saliva compounds than humans, which indicates for example that their digestion functions differently. Dogs' saliva is less acidic and can neutralize more acids (Pasha et al. 2018, Sanguansermsri et al. 2018). It also different levels of minerals such as calcium, potassium, and sodium (Feldman et

al. 2015). Dogs' initial breakdown of carbohydrates into simpler sugars is facilitated by pancreatic amylase, which is released into the small intestine. This is where most of the digestion and nutrient absorption occurs in dogs (Schermerhorn 2013, Feldman et al. 2015, des Gachons and Breslin 2016). Hence, dogs' saliva barely contains α-amylase to break down sugars, their saliva is a suitable target for measuring glucose levels because it is consistently present in their mouth (Gachons and Breslin 2016, Muñoz-Prieto et al. 2019).

In dogs, blood glucose passively diffuses into saliva across the intralobular ductular epithelium. As glucose from the blood transitions into saliva, it moves through the intercellular space filled with interstitial fluid. This fluidic environment acts as a medium through which glucose can move out of the cells and into the saliva (Schermerhorn 2013). Since the concentration of glucose in saliva is typically lower than in the blood, glucose moves from an area of higher concentration (blood) to an area of lower concentration (saliva), following the gradient caused by the difference in chemical concentration (Bagchi and Nair 2012).

Additionally, a study by Ioannou et al. (2021) observed an estimated blood-saliva glucose time lag of 30–40 minute, reflecting the pattern changes in salivary and blood glucose levels. This lag, along with high correlation times between salivary and blood glucose levels, suggests that despite the small sample size and high variability in salivary glucose levels within and between dogs, changes in salivary glucose are likely not random. These findings underscore the potential of saliva as a diagnostic tool for monitoring glucose levels and studying metabolic conditions in dogs (Ioannou et al. 2021).

1.2 Glucose homeostasis and metabolism

Carbohydrates are the primary macronutrient used in determining glucose levels after a meal (Muñoz-Prieto et al. 2019). Numerous studies (Farrow et al. 2013, André et al. 2017, O'Kell and Davison 2023) have found that consuming diets with high carbohydrate content lead to a rise in postprandial glucose and insulin levels in dogs. Persistent high insulin levels (hyperinsulinemia) are considered a significant factor in the development of diabetes in overweight pets. Pet foods containing excessive carbohydrates may challenge the body's ability to regulate glucose because diabetic pets have a disturbed insulin balance. Thus, high carbohydrate-containing foods pose a risk factor for diabetes mellitus balance management.

The postprandial state is commonly defined as the 6-hour period immediately following a meal. Conversely, after a fasting period of 14-16 hours, the body enters a postabsorptive state (Han et al. 2016, Dimitriadis et al. 2021). In the latter state the liver plays a crucial role in regulating glucose production through various pathways in glucose metabolism, including glycogenolysis, glucogenesis and glycolysis (Han et al. 2016).

Glucose homeostasis is crucial for providing a steady energy supply, especially to the brain and erythrocytes. In eukaryotic organisms' glucose is

stored in the form of glycogen in the liver and muscles (Figure 1). The levels of glycogen and glucose are regulated by hormones, such as insulin and glucagon, and this regulation adjusts according to the body's metabolic needs (Dimitriadis et al. 2021). Insulin prevails in energy rich states, promoting glycogen synthesis, while glucagon dominates in fasting conditions, stimulating glycogenolysis to release glucose into the bloodstream (Hantzidiamantis and Lappin 2023).

Glucose is a simple sugar derived from carbohydrates. It belongs to aldoses due to its aldehyde group. In an aqueous solution, glucose forms a ring structure, where the oxygen of the aldehyde group is connected to the hydroxyl group of the second-to-last carbon. Glucose is the main and critical source of energy for mammalian cells, which usually enters the body in isometric forms: monosaccharides, disaccharides or polysaccharides (Hantzidiamantis and Lappin 2023). During digestion, the intestines absorb sugars from food, converting them into simple sugars like glucose (Schermerhorn 2013). These sugars enter the bloodstream to reach all body tissues and cells.

Once glucose enters the energy-demanding tissues, it is metabolized through different pathways depending on the availability of oxygen. During glycolysis, which occurs in the cytoplasm, one molecule of glucose is converted into two molecules of pyruvate, with a net production of two molecules of ATP and two molecules of NADH. In the absence of oxygen, or under anaerobic conditions, the pyruvate is converted into lactate through a process called lactic acid fermentation. This conversion does not produce any additional ATP beyond the initial two molecules generated during glycolysis. However, when oxygen is present, or under aerobic conditions, each pyruvate molecule is transported into the mitochondria and enters the citric acid cycle (also known as the Krebs cycle). One molecule of glucose leads to two turns of the citric acid cycle, resulting in the production of an additional two ATP molecules directly. Moreover, the complete aerobic respiration of one molecule of glucose produces a total of about 30 to 32 ATP molecules, which includes ATP generated during the electron transport chain from the high-energy electron carriers (NADH and FADH2) produced during glycolysis and the citric acid cycle. Therefore, while the anaerobic pathway (glycolysis followed by lactic acid fermentation) yields only two ATP molecules per glucose molecule, the aerobic pathway (glycolysis followed by the citric acid cycle and electron transport chain) can generate approximately 30 to 32 ATP molecules per glucose molecule, making it a much more efficient process for energy production (Hantzidiamantis and Lappin 2023, Röder et al. 2016).

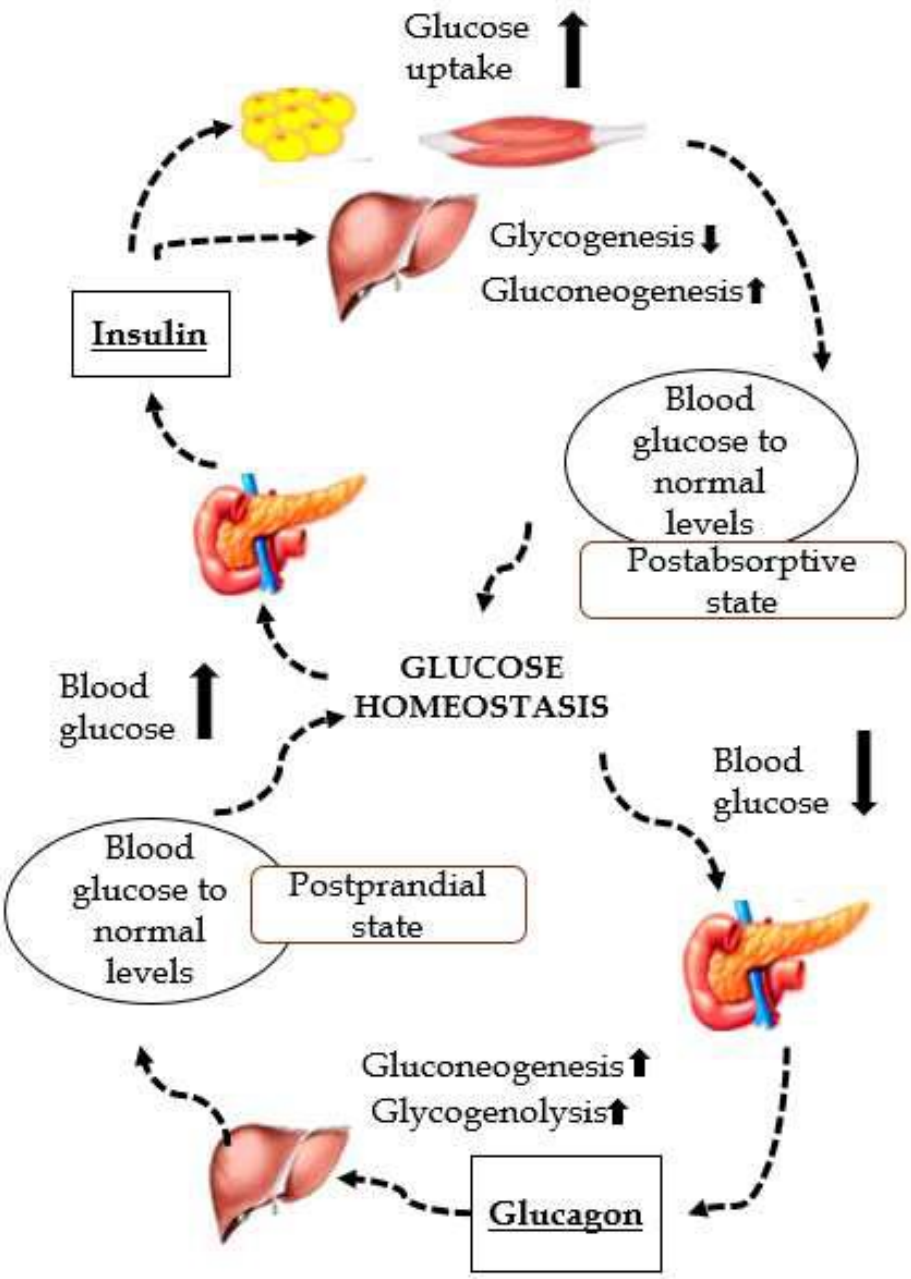


Figure 1. Regulation of blood glucose by insulin and glucagon. This diagram illustrates the hormonal control of blood glucose levels by the pancreas through the actions of insulin and glucagon. In response to low endogenous glucose levels, glucagon is secreted by the pancreas and initiates glycogenolysis, raising glucose in the blood. Postprandially, when exogenous glucose levels increase, insulin is released, facilitating glucose absorption by insulin-responsive muscle and fat tissues, and enhancing glycogenesis for glucose storage. Figure edited from Röder et al. (2016).

Glycogenolysis serves as the initial response to low blood glucose levels, where glycogen stores are broken down to maintain blood glucose levels (Han et al. 2016). In the postabsorptive phase, approximately 80% of blood glucose is released from the liver, with 50% attributed to glycogenolysis and the remaining 50% attributed to gluconeogenesis (Hantzidiamantis and Lappin 2023). The activity of glucogenesis continues to increase with prolonged fasting, constituting about 70% of glucose production after 24 hours and over 90% after 42 hours of fasting (Han et al. 2016).

Alternative energy metabolism pathways also exist in mammals. Animals with nutrition heavily depend on proteins as their main construction material and fats as their primary energy source utilize ketone bodies as their principal energy supply (Bagchi and Nair 2012). However, the primary emphasis of this thesis will revolve around glucose metabolism.

1.3 Glucose measurement methods

Glucose can be measured using various samples, including whole blood, plasma, serum or urine. Plasma and serum are preferred over whole blood because they provide more accurate readings, being about 15% higher due to the extra water in blood cells. These methods, however, are invasive as they require a certain amount of blood (Evans et al. 2023).

Traditional glucose measurement methods, which relied on glucose's reducing and condensing properties, were used in laboratories but faced issues like lack of specificity, toxicity, and cross-reactions with other substances. Modern methods in laboratories use enzymatic and hexokinase techniques due to their high accuracy, specificity, and minimal cross-reactions. For point-of-care and home monitoring, the enzymatic method is favoured for its simplicity and relative affordability (Knies et al. 2022).

In veterinary medicine, glucose measurement methods in dogs are similar to those used in humans. A recent advancement is the use of interstitial glucose, already a routine method in human medicine (Evans et al. 2023). The primary methods for glucose measurement in dogs include venous blood, capillary blood, and urine samples (Ismail-Hamdi et al. 2021). Although saliva sampling is considered a potential method, it has not yet been widely adopted in veterinary medicine. The normal fasting glucose levels in dogs across these sample types are presented in Table 1.

TABLE 1. Normal physiological ranges for glucose levels in different sample types in dogs. Table made by using information by Gupta and Kaur (2020), Ioannou et al. (2021), Knies et al. (2022) and Yadav et al. (2020).

Sample type	Normal range (mmol/l)
Interstitial Fluid	4.0-6.0
Urine	< 5.5
Whole Blood	3.9 – 6.0
Serum	4.0 - 6.0
Saliva	< 0.3

1.3.1 Blood glucose

Glucose and many other metabolic biomarkers are most concentrated in arterial blood. However, venous samples are usually used for laboratory analyses due to their accessibility. Both red blood cells and white blood cells have glycolytic enzymes that can consume glucose over time in a whole blood sample. To counteract this, a coagulation activator is commonly added to the sample tube, along with either EDTA or citrate to prevent clotting and fluoride is added to inhibit glycolytic enzymes, maintaining stable glucose concentration (Gurung et al. 2023).

There are two primary methods for measuring blood glucose levels in both humans and dogs. The first method involves obtaining a venous blood sample, typically from the vena saphena of the dog's front leg (Wess and Reusch 2000). The sample is then used for laboratory analysis to measure the glucose concentration using specialized equipment (Cook 2012). Two main categories of methods are used in these assays: enzymatic approaches, which include spectrophotometric assays, and non-enzymatic methods, such as High-Performance Liquid Chromatography (HPLC). The specialized equipment is primarily associated with enzymatic approaches and spectrophotometric assays. These methods are highly accurate and reliable for analysing blood glucose levels (Del Baldo et al. 2020). However, they are unsuitable for continuous glucose monitoring as they require a visit to the veterinarian for blood sampling (Del Baldo and Fracassi 2023), which can be challenging for fearful or restless dogs (Mott and Gilor 2023).

The second method uses a small drop of blood, typically obtained through a quick skin prick, and is measured using a handheld device called a glucometer (Luppa and Junker 2018). The test is performed by applying a drop of blood to a chemically treated disposable test strip designed for use with the glucometer (Mott and Gilor 2023). The functionality of glucometers relies on enzymatic reactions between glucose and specific enzymes embedded in the test strips, such as glucose oxidase or glucose dehydrogenases (Suchowersky et al. 2021). Depending on the glucometer's technology, this reaction generates a signal in the form of an electric current. The strength of this current is directly proportional to the concentration of glucose in the blood. The glucometer translates this reaction into measurable units, typically displayed in either millimoles per litre (mmol/l)

applied in Europe or milligrams per decilitre (mg/dl) in the United States (Gupta et al. 2017).

While glucometers are generally reliable for monitoring glucose levels in dogs, the accuracy can vary depending on the device (Wess and Reusch 2000, Gupta et al. 2017, Del Baldo et al. 2020). A point for concern is Suchowersky et al. (2021) study that found variance compared to venous blood glucose results, particularly at very high or very low glucose concentrations.

Blood samples for glucometer analysis in dogs are often obtained from the ear flap or paw pad (Jahan et al. 2023). Consequently, compared to venous blood sampling, this point-of-care device provides a more convenient and user-friendly method to measuring glucose levels (Moore et al. 2021). However, the glucometer can be unpleasant for dogs due to the discomfort associated with the needle prick needed to obtain the sample.

1.3.2 Urine glucose

Measuring glucose levels in urine is a common method for monitoring and diagnosing conditions such as glucosuria. Under normal circumstances, the kidneys filter glucose from the blood and reabsorb it back into the bloodstream, resulting in minimal amounts of glucose in the urine. A concentration below 1.67 mmol/l is considered a negative test result (Behrend et al. 2019). However, when blood glucose levels rise significantly, as in diabetes, the kidneys may not reabsorb all the glucose, leading to its excretion in the urine. A positive test result typically indicates urine glucose levels exceeding 5.5 mmol/l (1+ on test scales) (Yadav et al. 2020). However, there are exceptions to the test's reliability. When urine glucose levels are low (4 – 7 mmol/l) and acetoacetate levels are high (approximately 4 mmol/l), acetoacetate can interfere with the test, resulting in a false-negative (Zeugswetter and Schwendenwein 2020).

For optimal test performance, it is essential to collect a fresh urine sample in a sterile or clean container as close as possible to the time of analysis (Nelson 2015). Glucose degradation can occur if the sample is not analyzed within 1 to 2 hours post-collection. For longer preservation, samples should be refrigerated at 2-8°C, extending the viable analysis period up to 24 hours. Factors such as temperature, light, and bacterial growth can alter the urine sample composition, leading to incorrect results. For instance, high temperatures and bacterial presence can promote glucose breakdown, potentially underestimating glucose concentrations and affecting the assessment of a dog's diabetes mellitus status (Nelson et al. 2023).

While urine glucose measurement is a valuable diagnostic tool, it has limitations. It may not detect early or mild cases of diabetes, as glucosuria only occurs after blood glucose levels exceed the renal threshold (Behrend et al. 2019). Additionally, fluctuations in urine concentration throughout the day can lead to variability in glucose measurements, making it challenging to obtain consistent and reliable diagnostic information (Vientós-Plotts et al. 2018).

1.3.3 Interstitial glucose

The interstitial glucose monitoring technology enables a comprehensive analysis of glucose/ sugar levels in the interstitial fluid, which surrounds the body's cells. This fluid is an ideal sampling target as it closely reflects blood glucose levels, making it a reliable indicator of glucose metabolism. (Malerba et al. 2020). The Flash Glucose Monitoring System (FGMS), commonly known as FreeStyle Libre, represents a significant advancement in diabetes care for dogs. This technology allows for a detailed examination of glucose regulation by measuring sugar levels in the interstitial fluid, the fluid surrounding the body's cells (Knies et al. 2022).

The sensor's technology is based on the glucose-oxidase method. The sensor measures an electrical current proportional to the glucose concentration using an electrode with a lengthy carbon chain that houses both glucose oxidase and an osmium mediator, known as a 'wired enzyme.' After the glucose is reduced by glucose oxidase, the enzyme transfers its electrons to the osmium mediator instead of oxygen. The mediator then conveys these electrons to the electrode for measurement, eliminating the need for an oxygen-based reaction and a semipermeable membrane on the sensor (Del Baldo et al. 2020, Evans et al. 2023).

The detection limits of the sensor range from 1.11 to 27 mmol/l. The system becomes operational one hour after application, and factory calibration eliminates the need for additional calibration before or during the wearing period. The calibration factor is determined by measuring blood glucose and correlating it to the sensor's current at a specific point in time. Scanning the sensor with the reader provides instantaneous glucose readings within one second (Figure 2B) (Knies et al. 2022).

The FreeStyle Libre sensor is a compact, circular device measuring 35 mm by 5 mm. It contains a small catheter (0.4 mm by 5 mm) that is inserted beneath the skin to measure IG concentration. The sensor is designed to be worn comfortably for up to 14 days and is water-resistant (Corradini et al. 2016). Application of the sensor is straightforward and user-friendly, facilitated by a manufacturer-provided applicator. To ensure secure attachment, the sensor is typically placed in areas with minimal movement and adequate subcutaneous tissue, such as the lateral flank or the dorsal region of the neck (Figure 2A) (Howard et al. 2021). However, one of the challenges encountered in using the FreeStyle Libre sensor on dogs includes skin irritation and allergic reactions at the attachment site, as well as the potential for the sensor to become displaced or lost due to the dog's activities (Knies et al. 2022).

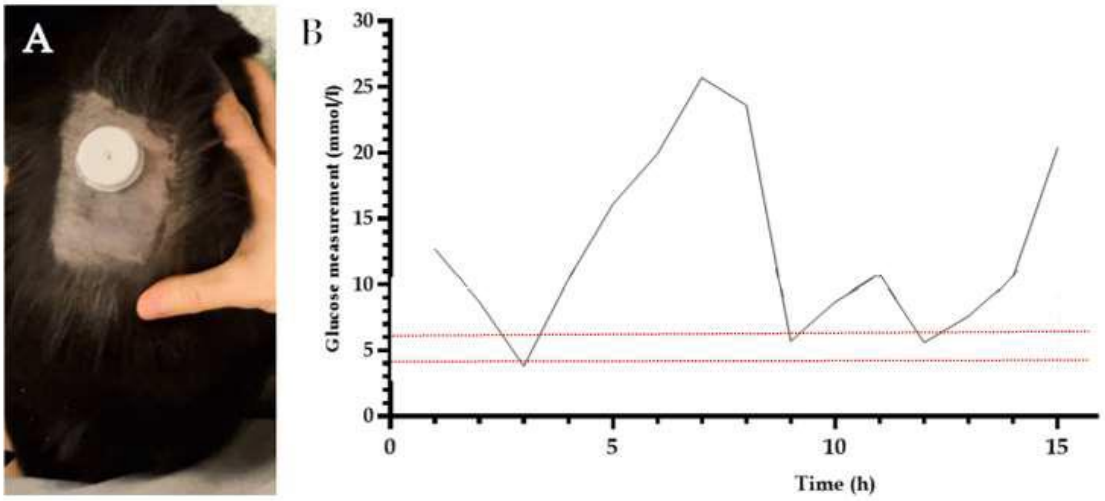


Figure 2. Continuous glucose monitoring in canines. Panel A shows a canine with a Free-style Libre 3 sensor attached to its neck, demonstrating the sensor's placement for an interstitial glucose monitor. Panel B shows a graphical representation of glucose spikes over time (X-axis in hours) and glucose measurements (Y-axis in mmol/l). The glucose curve depicts the dynamic changes in interstitial glucose concentration. The target range for normal interstitial glucose (4-6 mmol/l) is marked on the graph with a red dashed box.

1.3.4 Salivary glucose

One competitive and alternative method is measuring glucose from saliva. While salivary glucose levels are generally much lower, around 1-10% of blood glucose levels, Gupta and Kaur (2020) found a strong correlation between salivary and blood glucose levels in both healthy and diabetic dogs.

The mechanism behind salivary glucose testing is based on passive diffusion. Collecting saliva samples from dogs is relatively easy and can be done by pet owners, potentially increasing glucose monitoring compliance and allowing for more frequent testing (Ioannou et al. 2021). However, there are challenges with salivary glucose testing. Factors such as microbes in saliva, reduced saliva production, or collection issues can lead to inaccurate results. Additionally, individual variations and factors like diet, hydration, and oral health can influence the relationship between salivary glucose and blood glucose measurements. A significant limitation is the low concentration of glucose in saliva compared to blood (Lin et al. 2018). Despite these challenges, salivary glucose testing presents a promising alternative to invasive blood tests, offering a simpler and more comfortable method for monitoring glucose levels in dogs (Ioannou et al. 2021).

Interest in using saliva as a diagnostic fluid has grown due to its potential for measuring various substances like steroids, antibodies, hormones, and certain drugs accurately and easily. Additionally, the organic nature of saliva allows for

simple collection and preservation. Unstimulated saliva is preferred in testing to maintain consistent concentration and avoid pH changes (Gupta and Kaur 2020).

1.4 Statistical principles in medical research

The standard ISO 15197 provides the quality guidelines, requirements, and specifications that glucose measuring devices should comply with to guarantee their suitability for use. Many countries around the world use ISO's guidelines, through their national agencies, to assess whether each device is suitable for commercialization in their territory or not. However, exceptions exist, such as the United States, which has its own set of assessment guidelines. The ISO 15197 standard provides comprehensive quality guidelines, requirements, and specifications for glucose measuring devices. It sets criteria for accuracy, precision, and calibration to ensure reliable and consistent glucose readings. Additionally, the standard addresses user interface design, safety, and performance under various environmental conditions. It serves as a benchmark for manufacturers and regulatory agencies worldwide to assess the suitability of glucose monitoring systems for commercialization and clinical use. Understanding these metrics and standards is essential to comprehend why developers and researchers focus on certain technologies while leaving others behind, as well as the level of accuracy they intend to achieve. This standard also drives statistical analyses in medical research to ensure devices meet the necessary standards (Jendrike et al. 2017).

Various statistical principles in medical research provide the foundation for validating and interpreting data from diagnostic tests. Accuracy is the measure of a test's ability to correctly identify both positive and negative cases. Accuracy is expressed as a percentage, with a higher percentage indicating a more reliable test. Sensitivity, or the true positive rate, gauges a test's ability to correctly identify patients with the condition of interest. A highly sensitive test minimizes false negatives, thus ensuring that most patients with the condition are correctly diagnosed. Specificity, in contrast, is the true negative rate and measures a test's ability to correctly identify patients without the condition. A highly specific test minimizes false positives, ensuring that most healthy individuals are not misdiagnosed as having the condition. Positive Predictive Value (PPV) and Negative Predictive Value (NPV) provide additional context by reflecting the proportion of positive and negative results that are true positive and true negative results, respectively. These values are especially useful in the clinical context as they consider the prevalence of the condition in the population being tested. In addition to these, the F1-Score is a metric that balances the precision (PPV) and recall (sensitivity) of the test, providing a single score that gives insight into the test's overall performance (Monaghan et al. 2021).

Evaluating these metrics requires the use of various statistical methods, like Receiver Operating Characteristic (ROC). ROC curves plot the true positive rate against the false positive rate, showing the trade-off between sensitivity and

specificity across different thresholds (Zhou et al. 2009). In medical diagnostics, the interpretation of statistical metrics; accuracy, sensitivity, specificity can vary depending on the population being studied, the disease, the selected tests and the consequences of false positives or negatives. However, there are general benchmarks, often cited in the literature (Parikh et al. 2008, Monaghan et al. 2021, Zhou et al. 2009), that are presented in Table 2.

TABLE 2. This table summarizes the general benchmarks for interpreting the performance of medical diagnostic tests. Metrics are categorized into 'High', 'Moderate', and 'Low' based on their percentage values. These benchmarks are important for evaluating the effectiveness of diagnostic tests. Table done by Zhou et al. (2009) information.

Metric	**High**	**Moderate**	**Low**
Sensitivity/Specificity	80-100 %	60-80%	<60%
Accuracy	>90%	70-89%	<70%
PPV/NPV	>90%	70-89 %	<70%
F1-score	>0.80	0.50-0.79	<0.50
AUC for ROC curve	0.90-1.00	0.70-0.89	0.50-0.69

1.5 Aim of the study

The primary aim of this study was to evaluate the effectiveness and reliability of the Prototype 2 saliva glucose test as a non-invasive and rapid diagnostic tool for monitoring glucose levels in dogs. To achieve this, the study compared the effectiveness of the saliva glucose test against traditional methods of glucose measurement, including whole blood, serum, urine, and interstitial glucose measurement methods. Although previous studies (Gupta and Kaur 2020, Cui et al. 2022) had explored the correlation between saliva glucose concentrations and blood glucose levels in both dogs and humans, there was a notable gap in research regarding the relationship between interstitial glucose levels and saliva glucose levels specifically in dogs. Additionally, while there is a growing interest in non-invasive glucose monitoring methods for both human and veterinary medicine, there is limited research on the effectiveness and reliability of saliva glucose tests compared to traditional measurement methods in dogs.

It was hypothesized that the Prototype 2 saliva glucose test would yield a positive result when the blood, urine, and interstitial glucose levels are above the normal range levels (> 6 mmol/l) and negative results when the blood and urine glucose levels are below the normal range levels (<6 mmol/l). Additionally, it was hypothesized that the saliva glucose test stick would be effective in

determining glucose levels in dogs. The research questions guiding the study were:

1. Was the Prototype 2 saliva glucose test for dogs as effective as traditional urine and blood glucose tests in measuring glucose levels?
2. How accurately did the results of the Prototype 2 saliva glucose test correlate with interstitial glucose levels in dogs?
3. What are the dog owners' experiences associated with using the Prototype 2 saliva glucose test in monitoring their dogs' glucose levels.

2 MATERIALS AND METHODS

2.1 Study design

This study was done as a part of a larger research on canine metabolomics in the faculty of veterinary at the University of Helsinki in the DogRisk research group. The study was divided into three parts:

1. As a pilot study, we compared the salivary glucose test with traditional glucose measurement methods (whole blood, serum and urine)
2. The comparison of salivary glucose to interstitial glucose
3. The dog owners' experiences (Figure 3).

The study was designed this way to comply with ethical guidelines and laws for dog research. Owners conducted the saliva tests at home to avoid keeping the dogs in a clinical setting, which could cause stress and elevate glucose levels. Since diabetic dogs are a relatively small group in the dog population in the Helsinki metropolitan area, it was challenging to find candidates for this research. Because only ten dogs participated in the study, we couldn't yet compare the effectiveness of the test with ten measurements. To gather enough measurements, the study included the second part. This approach also helped us generate interest for future marketing and allowed the dogs to be in a familiar environment without the additional stress of a clinic.

The diabetic dogs were recruited for the study from the university animal hospital database Provet at the University of Helsinki and from the "Facebook-koirat" group. Non-diabetic dogs were recruited from friends and from researchers working in the DogRisk research group. The diabetic dogs had to have a previous diagnosis of diabetes mellitus and insulin treatment for at least 2 months before entering the research. The study was conducted in accordance with the appropriate ethical permits (ESAVI/452/2020).

In this study, there were two groups: five diabetic and five non-diabetic dogs. Due to fluctuating glucose levels in diabetic dogs, it was necessary to

compare diabetic and non-diabetic dogs as the latter have stable glucose levels. For this study it was important to include a diverse range of unrelated dogs to ensure variability in glucose concentrations; high, intermediate, and low levels. Without the inclusion of various glucose concentrations, the effectiveness of the saliva test cannot be proved.

Initially, biological samples (saliva, interstitial fluid, urine, and blood) and survey data were collected from four dogs between April and June 2023. Following the same methodology, another six dogs were sampled in December 2023. Since obesity in dogs is known to be a risk factor for diabetes and high glucose levels, the dogs' weight and body condition scores were also measured during the clinic visit.

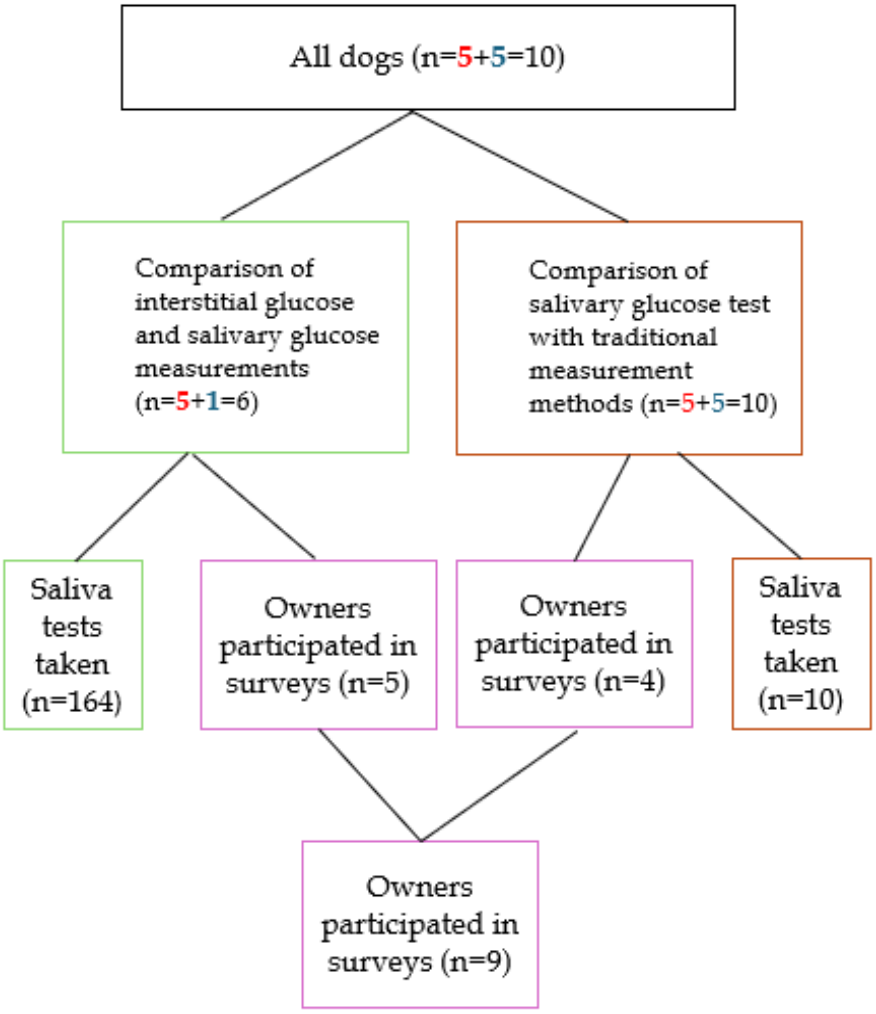


Figure 3. Study design flowchart. The numbers crossed out in red represent the count of diabetic dogs, and the numbers in blue represent the count of non-diabetic dogs.

2.2 Materials

2.2.1 Animals

All dogs underwent body weighing at the University of Helsinki in the Department of Small Animal Hospital (Helsinki, Finland). The dogs' body weight was measured using a veterinary-designed electronic platform balance (Kem EOS 150K100NXL, Germany). This platform balance was capable of measuring with a precision of 0.1 kg within a measurement range from 3 kg to 150 kg. The dogs' body weight was measured with an accuracy of 0.1 g. The body condition score (BCS) was evaluated both by the owners and my team during the clinic visit. The Hill´s classification BCS score that has a range from 1 to 5 was applied to assess the dog's conditions (Table 3).

TABLE 3. Hill´s classifications BCS scores

BCS 1	Emaciated or severely underweight
BCS 2	Underweight with visible ribs and minimal muscle mass
BCS 3	Normal weight with a healthy body condition
BCS 4	Overweight with excess body fat
BCS 5	Obese with a significant amount of excess body fat

In this study, there were dogs with different breeds. The breeds included: one Beagle, two Corgis, one Curly Haired Retriever, one Samoyed, and five mixed-breed dogs. All the dogs underwent a clinical examination and basic blood test to ensure their wellbeing before starting the study. Of all the participating dogs, four were castrated males, and six were females (5 neutered). The median age (years) of the dogs (n=10) was 8.5 +/- 3.17 (median +/- SD.) The median body weight (kg) for dogs (n=10) was 20.8 +/- 8.73 kg (median +/-SD.). The average BCS on a scale of 1-5 for dogs (n=10) was 3.7 +/- 0.9 (mean +/- SD.). These characteristics are summarized in Table 4.

TABLE 4. Characteristics of the dogs (n=10) for the study.

	n	Mean	Median	SD
Age (years)	10	6.27	8.5	3.17
Weight (kg)	10	19.36	20.8	8.73
Body condition score (scale 1-5)	10	3.7	3	0.9
Sex (female/male)	(6/4)			

2.3 Methods

2.3.1 Serum, whole blood and urine sample measurements

After a fasting period of 12-14 hours, serum samples were collected from all dogs. Owners brought in urine samples, and both fasted whole blood and saliva glucose levels were tested. Serum glucose sample were taken from the dogs' front leg cephalic or medial vein with a 20- or 22-gauge needle using a 10ml serum blood collection tube (BD vacutainer). The tubes contained a coagulation activator and a gel separator. After collection, the samples were allowed to clot over 10 min at room temperature. Then, within 30 minutes to 1 hour of collection, they were centrifuged at 3500 x g for 10 min at room temperature (~22°C). After centrifugation, samples were immediately transferred to 1.5 ml Eppendorf tubes (ThermoFisher) and analyzed using a KONELAB PRIME 60i analyzer (ThermoFisher Scientific, Vantaa, Finland). The analyser uses a photometric method relying on glucose oxidase, where glucose is oxidized to D-gluconate, producing an equal amount of hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide oxidatively couples with 4-amino antipyrine and phenol to form a red-colored quinonimine dye (Trinder colour reaction). The intensity of the red colour in the reaction is measured at 510 nm and is proportional to the glucose concentration in the serum sample. The excess serum that was not needed was frozen at -80°C for potential future analyses.

The whole blood glucose sample was collected from the identical measurement site used for the serum glucose method. The rapid glucose test was done using a point-of-care blood glucose monitor called Keto-Mojo™ glucometer (Abbott Laboratories). The monitor had a glucose strip with a tip. The strip was inserted into the meter and the tip was placed in the very small blood droplet (from 0.3 to 1 mikroL). The meter was held in the droplet until it beeped. Once the blood contacted the test strip, a reaction occurred between the blood and the specific enzymes on the strip. The meter was held in the droplet until it beeped,

followed by the glucometer translating the reaction into measurable units (mmol/l).

We measured glucose levels in urine samples that dog owners brought with them when they came for the initial testing and sample collection. The urine was collected in a clean container and instructed to be as fresh as possible. The samples were stored in the refrigerator (4-6°C) before analysis. Analysis was performed for the urine samples approximately 1-2 hours after collection using Multistix 10 SG (Siemens, Germany). Multistix 10 SG is equipped with 10 different pads, each designed to measure a specific compound from urine. However, in this study only the glucose pad was utilized. The Multistix 10SG was immersed in the urine sample and held for a few seconds before excess urine was absorbed from the edges of the stick. The stick was turned upside down onto an absorbent paper towel and the glucose results were interpreted at 30 seconds. The color change on the glucose pad was visually examined comparing it to a color scale provided by the manufacturer. The color change was determined by the chromogen oxidation process. Multistix 10SG measurement was read in mmol/l.

2.3.2 Interstitial glucose measurements

Freestyle Libre 3 glucose monitor was used to measure interstitial glucose levels. The monitor was installed for six dogs during a clinic visit, including five with diabetes and one without. To ensure that the Freestyle Libre 3 sensors provided accurate and reliable measurement results, they were placed on a clipped and sterile area (approximately 5 cm x 5 cm). More specifically, hair was removed from the installing area for optimal contact with subcutaneous tissue, followed by sensor attachment using a tissue adhesive (3M Vetbond Tissue Adhesive). The location of sensor attachment varied among the dogs; either the sensors were placed on the neck or shoulder area (three dogs) or positioned on the chest area (three dogs). To secure sensors stability throughout the 14-day monitoring period, various methods were used, including the use of tape, body bandages, dog shirts or human t-shirts. However, the number of monitors was limited because some of the sensors either came off the dogs or did not function properly. Yet, the owners actively participated in monitoring interstitial glucose using the Libre system when the sensor was properly attached.

2.3.3 Salivary glucose measurements

In this thesis the prototype 2 saliva test (Testi Technologies, Oulu, Finland) was used for measuring saliva glucose levels. The prototype 2 saliva glucose test strip has two pillows, one narrow and one wider, that are produced on selected substrate by fabrication with the bioink. The glucose tests were designed to detect as positive saliva glucose levels above 0.3 mmol/l, which is equivalent to 6 mmol/l of both blood glucose and interstitial glucose. The narrow pillow is a

control and turns greenish grey to confirm that the test is wet enough, while the wider is the glucose test turning green relatively fast (in about 10 seconds) if the glucose levels in the saliva are above the threshold level (0.3 mmol/l). The reaction is relatively faster with a high concentration of glucose in saliva and much slower with smaller glucose amounts. A gradient effect is observed, signifying that the colour intensity increases with higher concentrations of glucose. The saliva test is taken from dogs by touching or pressing the stick against the saliva on sublingual salivary gland on the dog's cheek. The saliva stick can be read 1 minute after taking the test and it indicates the positive (the test pad turns a shade of green) or negative (no colour reaction) result through a colour reaction (Testi Technologies, Oulu, Finland) (Figure 4).

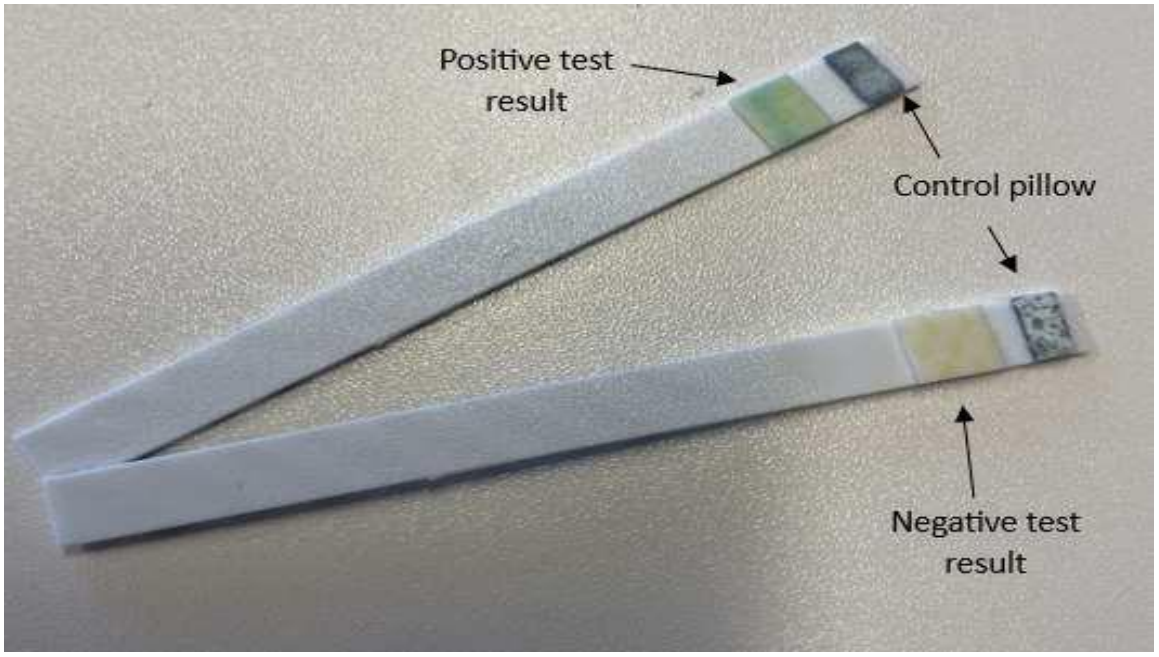


Figure 4. Prototype 2 saliva test outcomes: the upper test stick displays a positive result, evidenced by a colour transition to a greenish grey. Conversely, the lower test stick exhibits a negative result, as indicated by the absence of colour change in the test pillow. The control pillow on both strips have undergone a colour reaction, confirming sufficient moisture absorption, thus validating the test strip functionality.

Saliva sampling was done after the blood samples were collected. The saliva samples were collected from the dogs' cheek, between the space under or over the teeth rows and the inner side of the cheek, where the sublingual salivary gland is located. The results of the saliva samples were read approximately 1 minute after the collection, as stated in the manufacturer's instructions.

Owners monitored Freestyle Libre readings (mmol/l) to compare the effectiveness of the saliva test results to the interstitial glucose results. Additionally, allowing them to test whenever the glucose level deviated below 6 mmol/l or above 6 mmol/l, as indicated by the Libre sensor readings (Appendix 1). At the clinic visit, the owners received instructions on how to correctly

perform saliva tests at home and received 20 to 50 test strips for subsequent monitoring days. The saliva test's range for glucose levels is above 6 mmol/l classified as positive and below 6 mmol/l classified as negative.

2.3.4 Canine health and saliva test experience surveys

To comprehensively evaluate the usability and practical implications of saliva glucose testing from dog owners' perspectives, a detailed survey was made using REDcap (Research Electronic Data Capture). The survey was structured into two questionnaires tailored to extract a multifaceted understanding of the dog's health background and the owner's firsthand experiences with the saliva glucose test.

The first questionnaire was particularly designed to collect comprehensive background data on each participating dog. This included questions regarding the breed, sex, age, neuter status, extensive medical history, and diet. This detailed information was deemed essential for accurately assessing the demographics and characteristics of the canine population involved in this study (Appendix 2).

The second questionnaire was focused on gathering detailed feedback from the owners regarding their experiences with the saliva glucose testing. These encompassed questions designed to assess the ease of sample collection, the overall satisfaction with the testing process and the willingness of the owners to incorporate this testing method into their regular pet care routine (Appendix 3).

2.3.5 Statistical analyses

The statistical analyses were conducted to assess the diagnostic performance of the prototype 2 saliva glucose test in comparison to traditional glucose measurement methods. Calculations to assess sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), accuracy, and F1 score (Table 5) were performed and the results of saliva test versus traditional glucose measurement methods were compared. Receiver Operating Characteristic (ROC) curve analysis was applied to further investigate the discriminative ability of the saliva glucose test, with the Area Under the Curve (AUC) providing a measure of its overall performance in distinguishing between positive and negative interstitial glucose levels. All statistical analyses were performed using R (version 4.3.1). In R the analysis was performed with an open access statistical software package. The 'pROC' package was utilized for ROC curve analysis and diagnostic metric calculations. The limit of statistical significance was set at a p-value of <0.05, establishing a standard threshold for determining the meaningfulness of the observed associations and differences. All the collected results were presented in graphs and images that were generated with GraphPad Prism (version 10.2.1) and Microsoft Excel.

TABLE 5. Diagnostic metrics equations for sensitivity, specificity, PPV (Positive Predictive Value), NPV (Negative Predictive Value), accuracy, and F1 score. Here, TP stands for True Positive, FN for False Negative, TN for True Negative and FP for False Positive.

Metric	*Equation*
Sensitivity	$\frac{TP}{TP+FN}$
Specificity	$\frac{TN}{TN+FP}$
PPV	$\frac{TP}{TP+FN}$
NPV	$\frac{TN}{TN+FN}$
Accuracy	$\frac{TP+TN}{TP+TN+FP+FN}$
FI- score	$2\,X\,\frac{PPV+SENSITIVTY}{PPV\,x\,SENSITIVITY}$

3 RESULTS

3.1 Comparison of salivary glucose test with traditional measurement methods

To demonstrate the effectiveness of the saliva Prototype 2 test strip, a comparison was made with glucose level measurements from whole blood, serum, and urine. Four out of five diabetic dogs had above normal glucose levels in whole blood, serum and urine. The fifth diabetic dog had elevated level in serum and urine. All 5 non-diabetic dogs had normal glucose levels. The saliva test was positive for three of the diabetic dogs and negative for all non-diabetic dogs (Figure 4). The saliva test positive dogs had the highest glucose levels in serum and urine. The comparison of glucose measurement in diabetic and non-diabetic dogs is given in Appendix 4.

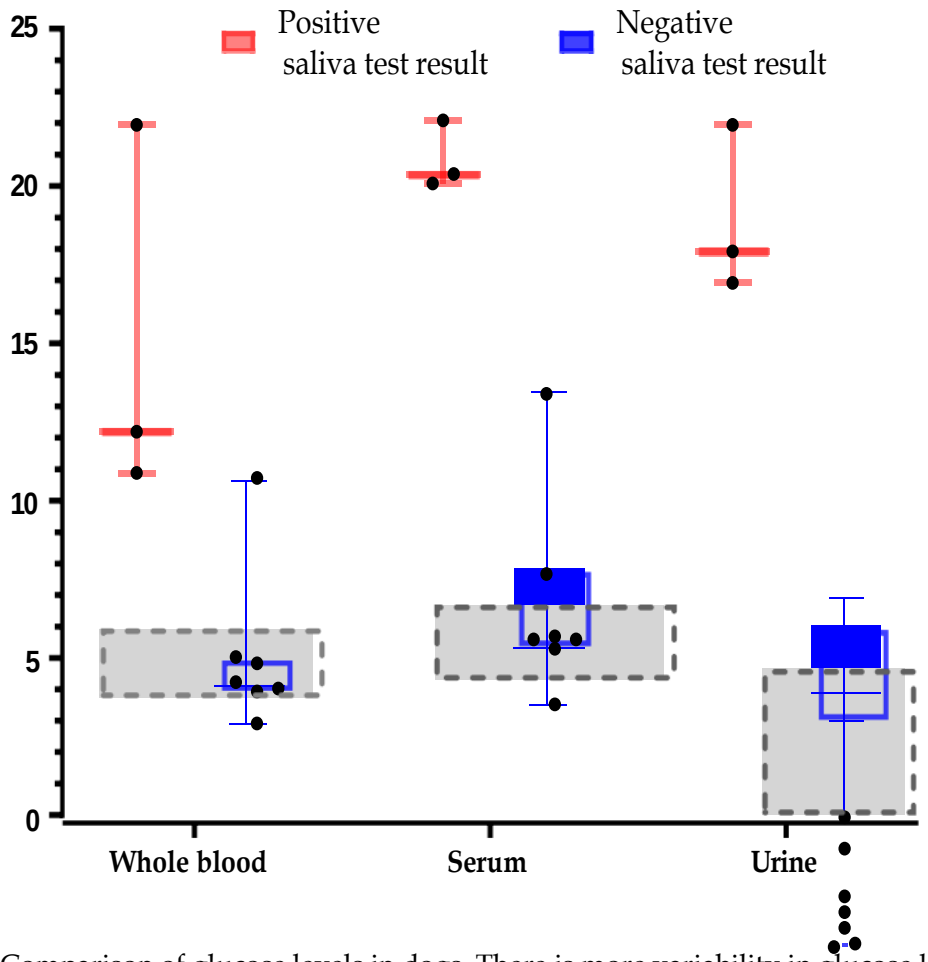
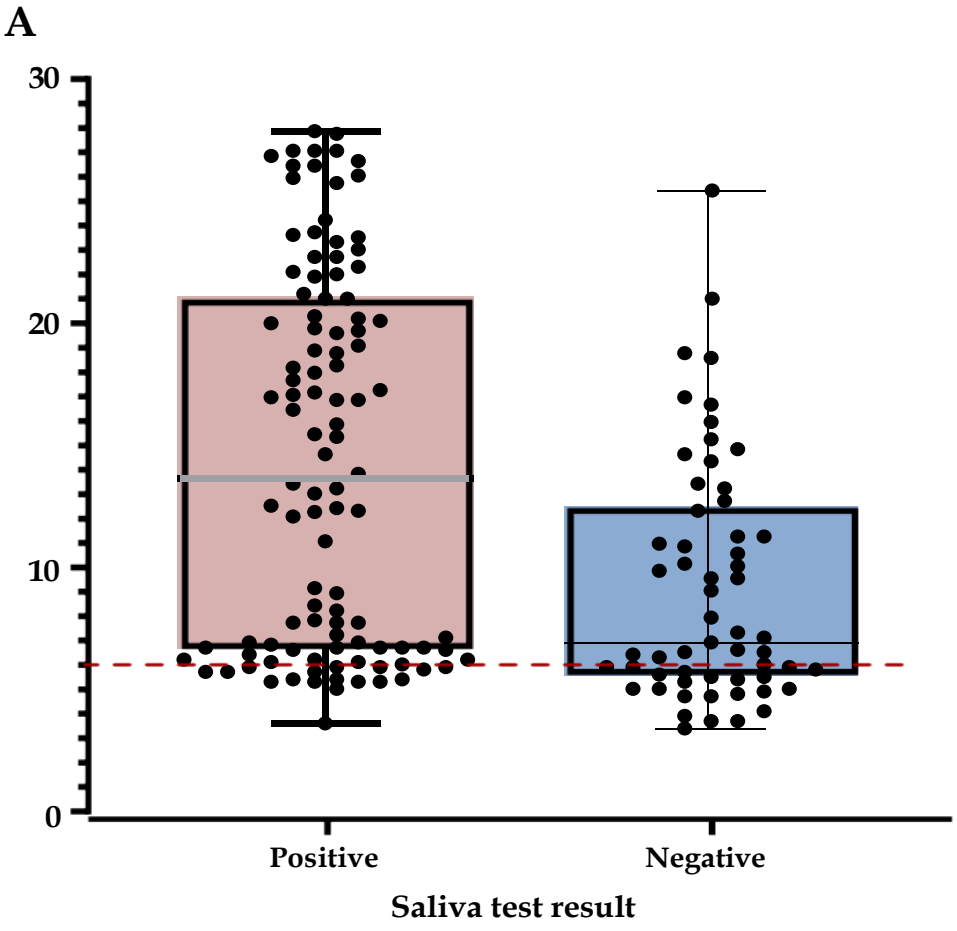


Figure 4. Comparison of glucose levels in dogs. There is more variability in glucose levels among diabetic dogs. This figure displays individual glucose measurements using different testing methods. Positive saliva test results are marked in red boxes, and negative results are marked in blue boxes. The grey boxes indicate the normal reference ranges for whole blood (3.9-6 mmol/l), serum (4 - 6 mmol/l), and urine (less than 5 mmol/l). All data points for each fluid concentration are displayed as black dots.

3.2 Comparison of interstitial glucose and salivary glucose measurements

The analysis of interstitial glucose (IG) levels in relation to saliva glucose test results was conducted to provide more measurement points and data for comparison with the saliva test outcomes. The analysis of interstitial glucose (IG) levels in relation to saliva glucose test results revealed significant differences between positive and negative outcomes across 164 tests. Out of the tests that were deemed positive, 11 of 106 had glucose levels within the normal range (4-6 mmol/l), but 95 tests resulted in higher ranges. Moreover, out of the 58 negative test results, 19 were within the normal glucose levels and 39 were out of the range. Dogs with positive saliva tests had an average glucose level of 14.27 mmol/l, while those with negative tests had an average of 9.1 mmol/l. This difference was also seen in the median values: 13.6 mmol/l for positive tests and 7 mmol/l for

negative tests (table 9 in appendix 4). The range of glucose levels was wider in the positive group (from 3.5 to 27.8 mmol/l) compared to the negative group (from 3.3 to 25.4 mmol/l). In this group of 164 tests, there were 12 false positive (7.2%) and 39 false negative (23.8%) results (Figure 5A). Out of all the saliva tests, 94 were true positive and 19 were true negative. The saliva glucose tests' overall accuracy was 71.3% (Figure 5B), and the FI-score was 0.841.



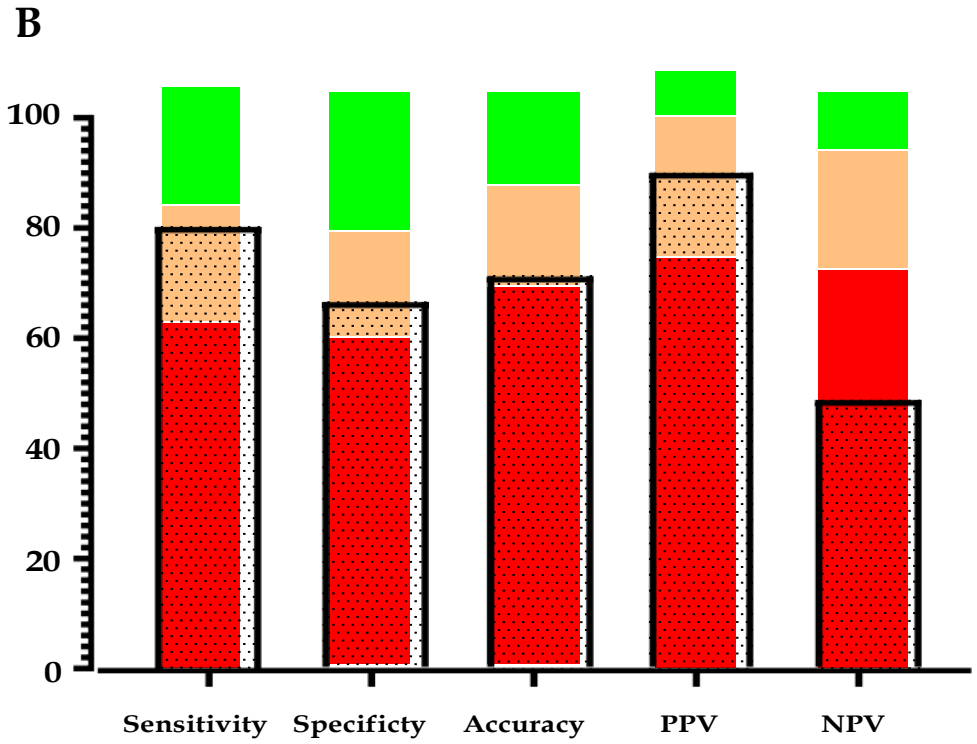


Figure 5. Diagnostic analysis of saliva glucose testing. Panel A shows the interstitial glucose concentrations corresponding to saliva test results. The red line at 6 mmol/l indicates the expected threshold distinguishing negative (below) from positive (above) saliva test outcomes. The line within the boxes (grey) represents the median values for each test outcome. The box represents the interquartile range (IQR), which includes the middle 50% of the data, with the lower and upper boundaries corresponding to the 25th and 75th percentiles (Q1 and Q3, respectively). The whiskers extend to the minimum and maximum values within 1.5 times the IQR. All data points for interstitial glucose concentrations are displayed as black dots. Panel B illustrates the diagnostic metrics of the saliva test, including sensitivity, specificity, overall accuracy, positive predictive value (PPV), and negative predictive value (NPV), shown as percentages. Red area showing the low rate, yellow area for moderate rate and green for high rate.

The ROC curve had an AUC value of 0.708 (Figure 6), suggesting that the test was moderately good at distinguishing between different glucose levels. The results indicate that while there is a strong relationship between saliva and interstitial glucose levels, the saliva test demonstrated variability in accuracy.

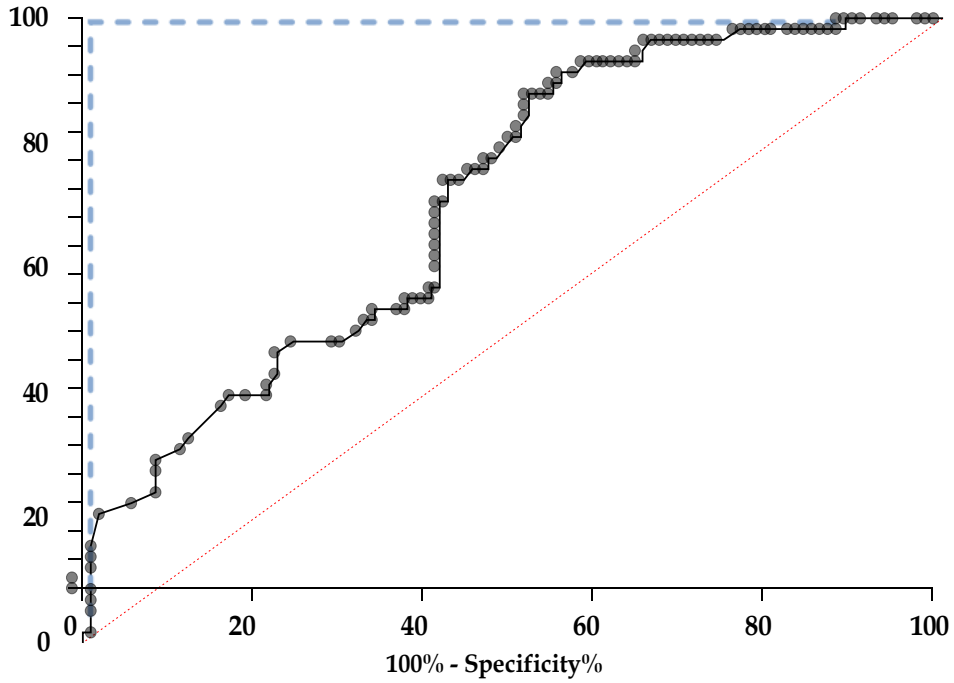


Figure 6. ROC curve analysis illustrating the accuracy of the saliva glucose test. The ROC curve demonstrates the performance of the saliva glucose test with an Area Under the Curve (AUC) of 0.708, indicating moderate accuracy. The curve illustrates the trade-off between sensitivity and specificity at different thresholds. The dashed blue line represents the ideal curve, where the test accuracy would be high.

3.3 Owners' experiences

Owners' experiences in using the saliva prototype 2 were collected to evaluate the potential user-friendliness of the test and to gauge interest for marketing purposes. This information is essential for understanding how the test might be received by users and its potential market appeal. Most of the dog owners (n=9) were positive about the usability and potential for home use of the saliva glucose tests (Figure 7). A significant 65% of respondents were willing to use the saliva test at home. Most owners found the test either 'okay' or 'easy' to use. However, 20% found it 'difficult', indicating a need to improve instructions or design for a better user experience. Opinions on interpreting the test results were mixed. While 40% found it 'neutral' in difficulty, another 40% found it 'somewhat difficult'. Interestingly, 75% said they would buy the test if it was available, showing strong market interest. The preferred places to buy the test varied, with 'veterinarians,' 'pharmacies,' and 'pet stores' mentioned, suggesting that selling the test through multiple channels could be effective.

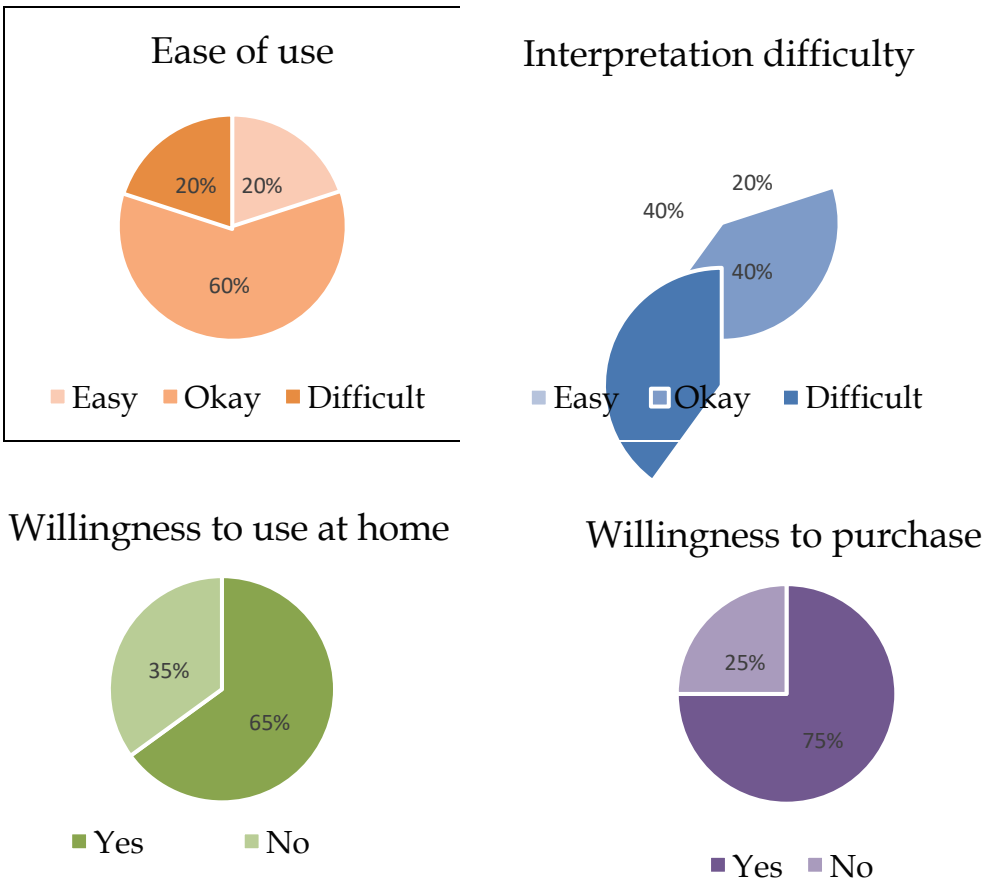


Figure 7. User feedback on saliva glucose tests. These pie charts represent user responses from a survey assessing the saliva glucose test. The charts illustrate participants' perceptions of the ease of use and interpretation difficulty of the test, as well as their willingness to use the tests at home and intention to purchase them for personal use. The data indicate that the majority find the tests easy to use and interpret.

4 DISCUSSION

In this stydy I tested the usability of a prototype saliva glucose test in the monitoring of diabetic dogs. Even though my initial pilot measurements showed that very high serum glucose levels could be picked up accurately with the prototype stick test performed in the clinic, moderate accuracy was observed when compared to real-time interstitial glucose sensor readings in home settings. However, additional product development is necessary to improve its precision and reliability.

The prototype 2 saliva glucose test demonstrated moderate sensitivity, specificity, accuracy and positive predictive value (PPV), indicating a likelihood that positive results are true positives. However, it showed poor negative predictive value, highlighting the need for caution when interpreting negative results. False negatives can significantly impact the reliability and effectiveness of diagnostic tools, potentially leading to the undermanagement or mismanagement of dogs' diabetes.

The prototype 2 saliva test presents a novel approach to saliva collection that diverges from traditional methods, which require the accumulation of saliva in a container, as demonstrated in the study by Damián et al. (2018). Instead, the prototype 2 test stick directly contacts the oral cavity's saliva, thereby streamlining the collection process and minimizing the risk of sample contamination or dilution. The direct-contact method bypasses issues linked to using external containers and stimulants for collection, thus likely decreasing false result chances by exposing the test interface only to dogs' saliva. However, insufficient saliva volume presents a notable risk for false negatives in glucose testing. Ensuring that the test pad is fully saturated is crucial for triggering the chemical reaction for glucose detection. If the saliva volume is inadequate, it might not initiate this reaction, emphasizing the importance of clear instructions for the collection process. Additionally, the extraction site within the oral cavity can affect the test's precision. Consequently, extracting saliva from an inappropriate location may yield a sample that inaccurately reflects the dog's glycaemic status, potentially leading to false results. Increased mucus in certain types of saliva, due to higher levels of mucopolysaccharides and glycoproteins, can impact the accuracy of glucose testing. These factors can increase the likelihood of false negative results in the saliva glucose test. This highlights the necessity for establishing and adhering to precise guidelines regarding the optimal sites for saliva collection in glucose testing.

Saliva composition can vary across different areas of the mouth due to factors like proximity to salivary glands and the presence of food residues. This variability means that test strips might interact differently with substances or surfaces in the oral cavity, potentially affecting the test results. For example, accidental contact with the tongue or other parts of the mouth that have enzymes

or food particles can interfere with the chemical reactions of the test. Saliva also naturally has a lower glucose concentration compared to blood. Additionally, the presence of oral microbes and elevated antimicrobial activity in saliva may cause an overestimation of glucose levels when using the oxidase–peroxidase method. These factors can influence the accuracy of the test, as pointed out in a study by Lin et al. (2018) and Gupta and Kaur (2020). Furthermore, contamination, such as the test strip touching the owner's hand or food, can also cause inaccurate results.

In our study, the results from home monitoring were notably weaker than those from the initial pilot conducted at the clinic. This could be due to various factors, such as incorrect saliva sample collection with the test stick, the dog having a dry mouth, or difficulty in obtaining a sufficient saliva sample. To improve this, clearer and more detailed instructions for saliva collection could be provided to the owners.

The moderate discriminative ability of the saliva test, indicated by an Area Under the Curve (AUC) value of 0.708, necessitates further optimization. Enhancing the test's sensitivity and specificity through technological advancements or integrating saliva glucose testing with other diagnostic indicators, such as insulin levels, glycated hemoglobin (HbA1c), and fructosamine, could improve its reliability and suitability for clinical applications. Future improvements in the test's sensitivity and specificity could include the use of advanced biosensors capable of detecting even subtle variations in glucose levels. Additionally, employing machine learning to analyze trends in glucose levels over time could enable precise differentiation between significant changes and background noise, significantly enhancing the test's accuracy.

The moderate accuracy and the challenges identified, including the presence of false positives and negatives, highlight areas for future research and development. Specifically, the tight range between median glucose measurements and their minimum and maximum values warrants attention. Although median values affirm the test's correlation with interstitial glucose levels, the limited range between the minimum and maximum is troubling. This suggests that the test has a restricted range of detection or possibly reflects a bias in our sample selection.

The prototype 2 saliva glucose test provided valuable insights into its practical application and acceptability among dog owners. Despite the overall positive reception, the study revealed some areas that require attention to optimize user satisfaction and test efficacy. One key area for improvement is the ease of test administration. A significant portion of the participants found the process only 'okay' or 'easy,' suggesting a need for clearer instructions or a more intuitive design to enhance user-friendliness. Additionally, the interpretation of test results presented challenges for some participants. A significant number found it somewhat difficult to interpret the results, highlighting the need for enhanced guidance or a simplified reading mechanism to reduce user anxiety and increase confidence in the test's outcomes.

Before the saliva test can be approved for commercialization, additional research and testing are essential to ensure its reliability. Given the relatively

small sample size utilized in this study, future research should aim to evaluate the saliva sticks across a larger sample size. Additionally, it would be beneficial to examine how different components of a dog's saliva might affect the functionality of the saliva test. Questions arise such as whether there is a component in the test pad that could interfere with glucose reaction, or if there are yet unexplored components in dog saliva that might influence the presence of glucose in saliva. Furthermore, it would be valuable to conduct a different type of test where glucose levels in saliva and blood or interstitial fluid are tested at shorter intervals, for example, every 10 minutes. This would improve the data quality and the interpretation of the results.

Indeed, while this study has focused on the prototype 2 saliva glucose test for canine diabetes, there's a compelling case for extending this diagnostic tool to feline patients. A few studies have reported a high prevalence of diabetes in cats (Feldman et al. 2015, Gottlieb and Rand 2018). Given that the physiological symptoms and biomarkers of diabetes are similarly present in cats, the potential utility of a non-invasive, saliva-based glucose test in feline diabetes management is considerable.

5 CONCLUSIONS

Insights into using a saliva glucose test for dogs' diabetes were provided by this study. Additionally, a non-invasive and easy test for dogs or canine diabetes monitoring is feasible in veterinary care. The test received positive feedback for its ease of use; however, clearer instructions and a simpler design are needed to enhance user satisfaction. The development of the prototype 2 saliva glucose test is a significant step towards less stressful diabetes monitoring in veterinary care. The prototype 2 saliva glucose test was found to be moderately accurate compared to the traditional methods. However, the test did not perform as effectively as the hypothesis had suggested. While the test has potential, more improvements are needed, particularly in enhancing its sensitivity and specificity. Future studies should focus on making the test more precise by deploying advanced biosensors capable of detecting subtle variations in glucose levels and by incorporating machine learning to analyse trends over time. Additionally, understanding factors that affect saliva glucose levels, such as diet, stress, and exercise, could further refine the test's accuracy and reliability.

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Jyväskylä April 19, 2024
Laura Tukonen

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APPENDIX 1. GUIDE TO FREESTYLE LIBRE 3 AND SALIVA GLUCOSE TESTING FOR PET OWNERS

FreeStyle Libre 3 glucose monitoring system & saliva glucose test collection instructions for pet owners

The FreeStyle Libre 3 glucose monitoring system is designed for measuring interstitial fluid glucose levels in dogs with diabetes. It aims to replace blood glucose measurement in monitoring diabetes treatment. The FreeStyle Libre 3 system includes a small sensor attached to the skin, which automatically measures glucose levels around the clock. The sensor is painlessly applied to the pet by a veterinarian during a clinic visit. Typically, dogs do not notice the sensor's application or its presence during the measurement period.

Before the Clinic Visit:

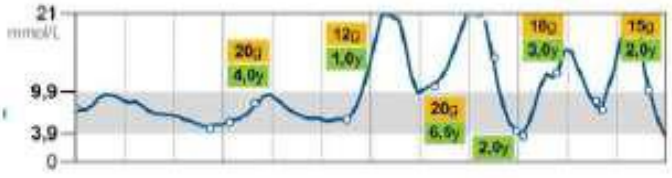
- Create a FreeStyle Libre account: https://www.freestyle.abbott/fi-fi/myfreestyle/sign-up.html
- Download the FreeStyle Libre 3 - FI app from the App Store (Apple) or Google Play (Android) and log in.

At the Clinic:

- We will test the connection's functionality before you go home (connecting takes about 60 minutes).
- The "clinic code" will be entered at the clinic, allowing us and your veterinarian to monitor the glucose curves online.

Saliva Test and Glucose Spike Monitoring Instructions:

If you observe unusually high (over 6 mmol/l) or low (under 6 mmol/l) glucose values in FreeStyle Libre measurements, try the new saliva stick method to check glucose levels at the same moment. This helps to evaluate the effectiveness of the saliva stick method at these exceptionally high or low glucose levels.



Example of a glucose curve produced by freestyle libre (note! each dog's glucose curve is individual).

- **If the glucose level shows above 6 mmol/l, take a saliva test → The saliva test should be positive.**

- **If the glucose level shows below 6 mmol/l, take a saliva test → The saliva test should be negative.**

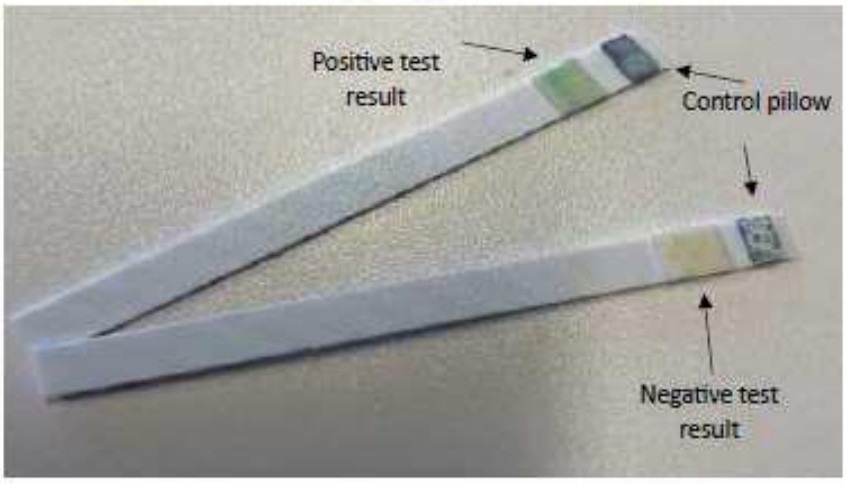
Record the results (libre + saliva test) on the form (provided during the clinic visit) along with insulin doses and the amount and type of food eaten.

If your dog eats twice a day, take at least four saliva tests per day from it before and after (30 minutes after) meals.

Test interpretation:

- Control pad = upper (smaller pad) → Indicates if the stick is sufficiently wet (if there is enough saliva; if the control pad does not change colour, try to collect more saliva).
- Sample pad= Lower (larger pad) → indicates the amount of glucose in the saliva.
- Positive result= green colour reaction
- Negative result= no colour reaction

TAKE A PHOTO OF THE SALIVA STICK RESULT (about 1 minute after taking the saliva test) AND SEND the photos and the result form via email/WhatsApp/chat.



Other Instructions:

- The dog should avoid swimming/bathing while the FreeStyle Libre sensor is in use.
- If the dog is to have an MRI, the sensor must be removed.
- If the sensor comes off, contact (phone number….).

Removing the Sensor from the Dog: The sensor should stay on the pet for a maximum of 14 days. A used sensor cannot be reused. You can remove the sensor yourself (often the "adhesive" side of the sensor easily detaches from the dog's skin like "taking off a plaster"). A dent may remain where the sensor was applied, which usually disappears within a few days. The sensor can be disposed of in mixed waste.

APPENDIX 2. CANINE HEALTH SURVEY

Thank you for participating in our study. Please fill out the questionnaire below.	
By responding to this questionnaire, you consent to your responses being used in research conducted by the University of Helsinki. Please sign your consent electronically with "add signature".	_____
Information about your dog	
Your name and your dog's name (separate them with a comma)	_____
Gender	◯ Female ◯ Male
Neutered/Spayed	◯ Yes ◯ No
Age	_____
Breed	_____
Feeding habits	
Describe what your dog ate yesterday. State the amounts either in percentages or fractions (e.g., 1/5) and how often it usually receives this food. Was this meal typical for your dog?	_____
What food does your dog eat?	☐ Dry food (knibble) ☐ Wet food ☐ Raw food ☐ Home cooked
If your dog eats something else, what is it?	_____
How many times a day does your dog eat?	◯ 1x a day ◯ 2x a day ◯ 3x a day ◯ More than 3x a day ◯ Always available
What kind of exercise do you usually do regularly with your dog? (Regular means more than 3 days a week.)	☐ Light exercise (e.g., walking) ☐ Moderate exercise (e.g., playing in the dog park) ☐ Intense exercise (e.g., running/cycling/active hobby with the dog)

Has your vet diagnosed your dog with any disease? If yes, what disease is it?	----------
Is your dog currently suffering from anything?	◯ Yes ◯ No ◯ Don't know
If yes, what?	----------
Has your dog been diagnosed with diabetes?	◯ Yes ◯ No
If yes, what insulin does your dog receive (mention the brand name)? How large is the dose (how many times a day)? What is the strength of the insulin (mg/ml)? What time does your dog receive its medication?	----------
What is your dog's usual morning fasting glucose level? (mmol/l)	----------
Do you think your dog's diabetes management is in balance?	◯ Yes ◯ No ◯ I don't know

Stress Factors

We ask about possible stress factors for your dog as they can affect your dog's blood sugar levels.

Do you feel your dog often experiences stress? Factors causing stress can include a family member moving out, a new family member joining the household (such as a newborn baby or a new spouse), a family member's illness, or the whole family moving.	◯ Yes ◯ No ◯ I don't know
What type of housing do you live in?	◯ Apartment ◯ Row house/semi-detached house ◯ Detached house ◯ None of the above
How many hours a day is your dog home alone?	◯ 0-4h ◯ 5-10h ◯ 11-14h ◯ More than 14h
Do you think the living environment is peaceful for the dog?	◯ Yes ◯ No

APPENDIX 3. OWNERS' EXPERIENCES SURVEY

Please fill out the questionnaire below. Thank you!	
Your name and your dog's name (separate them with a comma)	_____
How did you feel about performing the test?	◯ Very difficult ◯ Difficult ◯ Just okay ◯ Okay ◯ Easy
If it was difficult, what made it so?	_____
Do you think the test is easy to interpret?	◯ Very easy ◯ Moderately easy ◯ Neither easy nor difficult ◯ Moderately difficult ◯ Very difficult
Could you imagine performing a saliva test on your dog at home?	◯ Yes ◯ No
Would you buy the test if it were available for sale?	◯ Yes ◯ No
If you would buy the test, where would you purchase it from?	◯ From a veterinarian ◯ From a pharmacy ◯ Online/from an online store ◯ From a pet store
How much would you pay for a single test stick?	_____
Do you have any suggestions for improvements to the manufacturer?	_____

APPENDIX 4. GLUCOSE MEASUREMENT RESULTS

TABLE 6. Glucose measurements in whole blood. This table compares the glucose measurements in whole blood between diabetic and non-diabetic dogs. It includes mean, standard deviation (SD), median, and the range (minimum/maximum) of glucose levels in mmol/l.

Whole blood		
Metric	Diabetic dogs (n=5)	Non-diabetic dogs (n=5)
Mean (mmol/l)	10.62	3.92
SD	7.39	0.68
Median (mmol/l)	10.70	4.00
Min./max. (mmol/l)	3.9/22.0	2.9/4.8

TABLE 7. Glucose measurements in serum. Table B presents the serum glucose measurements, detailing mean, SD, median, and range of glucose levels for both diabetic and non-diabetic dogs.

Serum		
Metric	Diabetic dogs (n=5)	Non-diabetic dogs (n=5)
Mean (mmol/l)	12.62	5.02
SD	7.58	0.16
Median (mmol/l)	12.80	5.10
Min./max. (mmol/l)	4.7/20.3	4.8/5.2

TABLE 8. Glucose measurements in urine. This table shows the urine glucose measurements, featuring mean, SD, median, and glucose level ranges in mmol/l for diabetic versus non-diabetic dogs.

Urine		
Metric	Diabetic dogs (n=5)	Non-diabetic dogs (n=5)
Mean (mmol/l)	11.64	5.02
SD	9.16	0.16
Median (mmol/l)	14.0	2.6
Min./max. (mmol/l)	2.9/21.8	1.8/2.2

TABLE 9. Statistical summary of interstitial glucose levels. The table summarizes median, mean, minimum, and maximum values according to the saliva test (SG) results: positive n=106 and negative n=58.

	SG results (n=164)	
Metric	Positive (n=106)	Negative (n=58)
Mean (mmol/l)	14.2	9.1
SD	7.6	4.9
Median (mmol/l)	13.6	7.0
Min./max. (mmol/l)	3.5/27.8	3.3/25.4