

Development of a preliminary clinical approach for detecting insulin resistance for normoglycemic control in canine diabetes

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Abstract

Consistent and effective blood glucose (BG) control is crucial in the treatment of diabetes mellitus (DM) and insulin resistance (IR). In this study, the surrogate indices for monitoring insulin sensitivity and the preliminary clinical approach for detecting IR were established. Fasting serum C-peptide was measured by a commercial enzyme-linked immunosorbent assay (ELISA) kit and utilized as an endogenous insulin representation. The homeostasis model assessment of insulin resistance (HOMA-IR) was then calculated. To detect the IR, the relationship between HOMA-IR and fasting serum C-peptide in each individual subject was analyzed, and designed cut-off values. The results revealed that the HOMA-IR of the DM group was significantly higher than that in the normal group ($p < 0.001$). Based on the designed cut-off values, the preliminary clinical approach for detecting IR (CUVET DM-IR grid I) was proposed. The subjects could be categorized based on CUVET DM-IR grid I into four categories: 1) non-IR DM group, 2) IR DM group or non-IR DM group with the risk of IR development, 3) normal group, and 4) uncategorized group. When combined, a preliminary clinical strategy for detecting IR based on CUVET DM-IR cut-off values may be considered and applied in cases of newly diagnosed diabetes, uncontrolled diabetes, and other underlying factors. In diabetic situations, the therapy intervention for insulin sensitivity enhancement may play a supporting role in achieving normoglycemic management.

Keywords: diabetes mellitus, HOMA-IR, C-peptide, insulin resistance

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Introduction

Diabetes mellitus (DM) is a common endocrine disorder in dogs, characterized by defects in insulin production, insulin action, or both (Rand, 2020). Beta cells, located in the pancreas, play a crucial role in producing insulin. Insulin is the hormone responsible for regulating blood glucose levels by facilitating the uptake of glucose into cells for energy or storage. Clinical indications manifest as standard, such as polyuria, polydipsia, polyphagia, and weight loss (Fall *et al.*, 2007). Fasting hyperglycemia (blood glucose; BG above 180-220 mg/dL) is used as a criteria for tentative diagnosis of a diabetes case (Nelson and Reusch, 2014; Behrend *et al.*, 2018). Glycosuria is commonly observed when BG exceeds the renal threshold of 180 mg/dL in dogs (Watanabe *et al.*, 2004). DM is classified into two categories according to the criteria related to the pathophysiology of the disease: type 1 DM and type 2 DM. Type 1 DM, a typical DM in dogs formerly known as insulin-dependent DM or IDDM, is characterized by permanent hypoinsulinemia caused by pancreatic beta-cell destruction (Rand *et al.*, 2004). Type 2 DM is formerly known as non-insulin dependent DM or NIDDM. This DM type is characterized by the defects of insulin action (insulin resistance; IR) and/or insulin secretion. Prolonged or uncontrolled disease may lead to the progression of Type 2 DM to Type 1 DM, often resulting from beta-cell dysfunction or destruction over time. Diabetic ketoacidosis (DKA), a life-threatening complication of diabetes mellitus (DM), is found in uncontrolled hyperglycemic cases from a lack of insulin. Cells are then unable to uptake glucose for cellular energy, resulting in ketone production from lipid metabolism (Feldman and Nelson, 2004).

Consistent and effective BG control is crucial for diabetes treatment. However, numerous factors can influence the success of normoglycemic control. One such factor is the efficiency of the clinical approach for screening and categorizing the first-diagnosed and ongoing-treated diabetes cases based on their ability in insulin response. When the cell's response to the action of insulin is diminished or reduced, IR occurs. It causes numerous metabolic disturbances, including type 2 DM and cardiovascular diseases (Paz and Vizmeg, 2013). The causes of IR in dogs include Cushing syndrome, diestrus, and obesity (Fleeman and Barrett, 2023). Many approaches have been used for the detection of IR in human diabetes patients e.g. defining insulin sensitivity index (ISI) and hyperinsulinemic-euglycemic clamp testing (De Koster *et al.*, 2016). However, some approaches are time- and budget-conscious (De Koster *et al.*, 2016). The use of homeostasis model assessment of insulin resistance (HOMA-IR) as an ISI and IR detection platform has been studied and proposed as one of the interesting approaches (Borai *et al.*, 2011). The HOMA-IR utilizes two primary parameters for analysis: secreted endogenous insulin and fasting blood glucose concentration (Kawaguchi *et al.*, 2004). The early established HOMA-IR calculator incorporates a number of co-analysis factors aimed at determining the best predictive ISI parameter for IR detection, such as the quantitative insulin sensitivity check index (QUICKI) and the revised quantitative insulin

sensitivity check index (RQUICKI) (De Koster *et al.*, 2016). For IR detection, the quantification of C-peptide was used as an endogenous insulin representative for continuous monitoring (Fall, 2009; Jones and Hattersley, 2013). However, the calculator was mainly invented for human application.

As mentioned previously, the early detection of IR is very useful for adjusting the therapeutic approach since the exogenous insulin treatment is no longer effective in such circumstances. Other treatment approaches to enhance insulin sensitivity are indeed necessary. In human diabetes care, several oral hypoglycemic drugs can enhance insulin sensitivity and help in the achievement of long-term normoglycemic control (Snyder and Berns, 2004). Metformin, a biguanide hypoglycemic drug, illustrates the blood glucose-lowering effects of the reduction of endogenous glucose production from two pathways: decreased glycogenolytic process and increased insulin sensitivity in peripheral tissues (Chu *et al.*, 2000; Saini and Yang, 2017).

The existence of IR in canine diabetes patients is not clearly detected due to a lack of available screening methods, and the gold standard, hyperinsulinemic-euglycemic clamp, is time-, budget-consumed and rarely employed in practice. However, this information is necessary to increase the efficiency of clinical glycemic control. Thus, the goal of this study was to design a clinical approach for IR detection and to conduct preliminary testing of intervention as an on-top metformin treatment in canine diabetes.

Materials and Methods

Animals: The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC, 1731021), Faculty of Veterinary Science, Chulalongkorn University. All subjects were enrolled from the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University, with owner informed consent. Seven dogs were healthy, with no systemic disorders and fasting blood glucose levels below 180 mg/dL (normal group). Eighteen dogs were diagnosed with diabetes (DM group). Fasting BG above 180 mg/dL, glucosuria, and the clinical signs with polyuria, polydipsia, polyphagia, and weight loss without the presence of DKA and systemic diseases were used as inclusion criteria.

One canine diabetic pilot case was enrolled. The dog had poorly controlled diabetes by using exogenous insulin and was categorized as IR DM group or non-IR DM group with the risk of IR development of CUVET DM- IR grid I. The clinical indications displayed polyuria, polydipsia, polyphagia, and weight loss.

Blood collection: Two mL of blood was drawn from a cephalic or saphenous vein under an aseptic technique. One drop of blood was immediately used for BG testing. After being transferred to a plain collection tube, the blood samples were processed for serum collection. Briefly, samples were centrifuged at 2,460 g, 7°C for 20 min. The serum was then collected and stored at -80°C for further analysis. Serum collection was done within two h following blood collection.

Serum C-peptide concentration analysis: The concentration of C-peptide in serum was evaluated using a commercial enzyme-linked immunosorbent assay (ELISA) kit for human C-peptide (with canine cross-reactivity) following the manufacturer's procedure (Merck Millipore, USA). In brief, an immobilized monoclonal antibody (mAb) was used to capture the sample C-peptide in a microtiter plate. After unbound washing, another biotinylated mAb against C-peptide was applied, followed by streptavidin conjugated with horseradish peroxidase (HRP). The HRP substrate tetramethylbenzidine (TMB) was then added. At OD450, the enzyme activity was measured. The concentration of C-peptide was estimated using parallel standard curves.

Metformin administration: Metformin was administered in one pilot canine diabetic case. Metformin solution compounding (50 mg/mL) was used for one month at a dose of 5 mg/kg twice a day. The medication was given half an hour before the meal. During the study, the insulin dosage was reduced by half compared to the previous prescription. One month of treatment with exogenous insulin and metformin was required.

Insulin sensitivity surrogate indices: Fasting serum C-peptide and HOMA-IR were used as the surrogate ISI. HOMA-IR was calculated according to the following formula: $\text{HOMA-IR} = \text{fasting BG (mg/dL)} \times \text{fasting serum C-peptide concentration (ng/mL)}$ (De Koster et al., 2016).

Statistical analyses: The normality of the data was checked using the Shapiro-Wilk ($p > 0.05$). For normal distributed data, data was shown as mean $\pm$ standard deviation (SD). The non-normal distributed data, data was shown as median and data range. The nonparametric and parametric data were analyzed using the Mann-Whitney U test and ANOVA, respectively. Statistical difference was recognized when p -value < 0.05 . All statistical analyses were performed using SPSS software (SPSS v.22 for Windows, IBM, USA).

Results

Surrogate indices for monitoring insulin sensitivity: Endocrine function of the pancreas was tested by quantifying fasting serum C-peptide using ELISA. The fasting serum C-peptide concentrations of normal and diabetic dogs were summarized as follows. The minimum, maximum, and mean of fasting serum C-peptide concentrations in the normal group were 0.31, 0.90, and 0.56 ± 0.20 ng/mL, respectively. The result of fasting C-peptide concentrations in the DM group was not normally distributed, the lowest concentration was 0.27 ng/mL, the highest concentration was 2.85 ng/mL, and the median

concentration was 0.65 ng/mL. Fasting serum C-peptide concentrations between normal and DM groups were not significantly different (Fig. 1).

The surrogate index for monitoring insulin sensitivity was represented as the HOMA-IR. The calculation of HOMA-IR was described earlier. The ranges of HOMA-IR in normal and DM groups were 30-61 and 59-738, respectively. The normal and DM groups had HOMA-IR averages of 48.7 and 216.67, respectively. HOMA-IR of the DM group was significantly higher than that in the normal group ($p < 0.001$). Box plots of the HOMA-IR are shown in Fig. 2.

Preliminary clinical approach for detecting insulin resistance (CUVET DM-IR grid I): To establish the preliminary clinical approach for detecting the presence of insulin resistance in diabetes case (CUVET DM-IR grid I), the relationship between HOMA-IR and fasting serum C-peptide concentration in each individual subject was analyzed and plotted (Fig. 3). The cut-off values for categorizing the subjects were set (Fig. 3). In this regard, three cut-off values were used i.e., a) the average fasting serum C-peptide concentration in the normal group, b) the mean+2SD of fasting serum C-peptide concentration in the normal group, and c) any C-peptide level multiplied by the normal limit of fasting blood glucose concentration (120 mg/dL) (Sharma and Jain, 2014).

Based on the designed cut-off values, each individual subject could be categorized according to the relationship between HOMA-IR and fasting serum C-peptide concentration into four categories: non-IR DM group (area A and B), IR DM group or non-IR DM group with the risk of IR development (area C), normal group (area D and E), and 4) uncategorized group (area F) (Fig. 3).

To confirm the grouping criteria, ANOVA was used to analyze the differences among data in A, B, and C areas. The results showed that the data in the C area was significantly different from the other groups ($p < 0.001$). No difference was found between the data in A and B areas ($p > 0.05$) (Table 1). According to statistical analysis, the data in the C area was assigned to be IR DM group or non-IR DM group with the risk of IR development, while data in A and B areas were assigned to be non-IR DM group.

Improving insulin sensitivity for glycemic control in a pilot canine diabetes case: To exert glycemic control in canine diabetes, the concept of insulin sensitivity enhancement was studied by using an on-top metformin treatment along with exogenous insulin administration. The results showed that the fasting blood glucose concentration was reduced from 367 mg/dL to 339 mg/dL, and the fasting serum C-peptide concentration was increased from 0.65 ng/mL to 0.9 ng/mL. The clinical signs did not differ between before and after treatment.

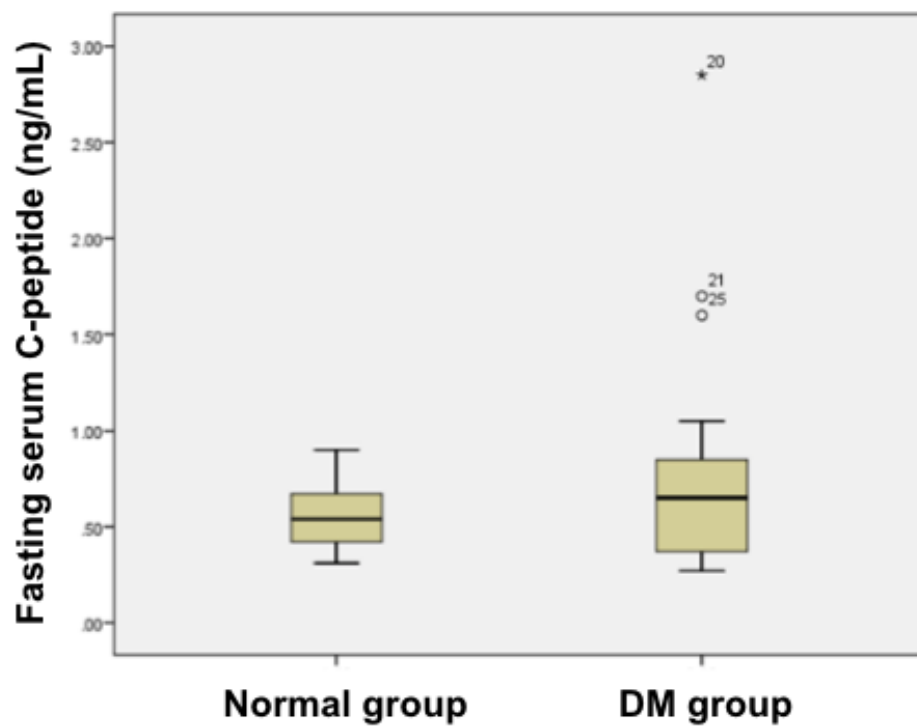


Figure 1 Boxplot of fasting serum C-peptide concentrations (ng/mL) in the normal group (n = 7) and DM group (n = 18).

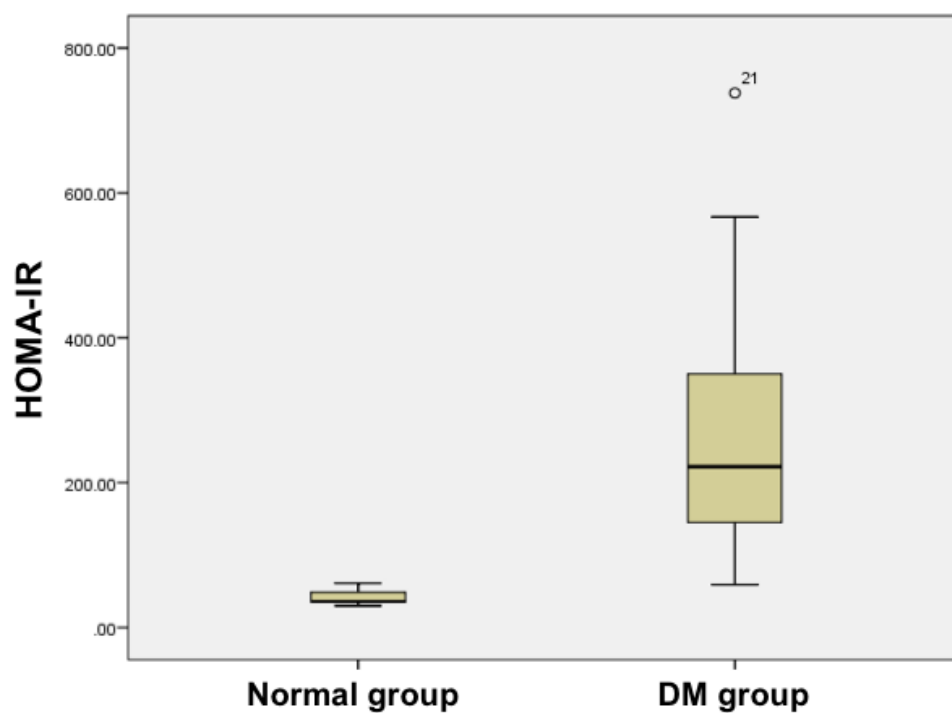


Figure 2 Box plot of HOMA-IR in the normal group (n=7) and DM group (n=18).

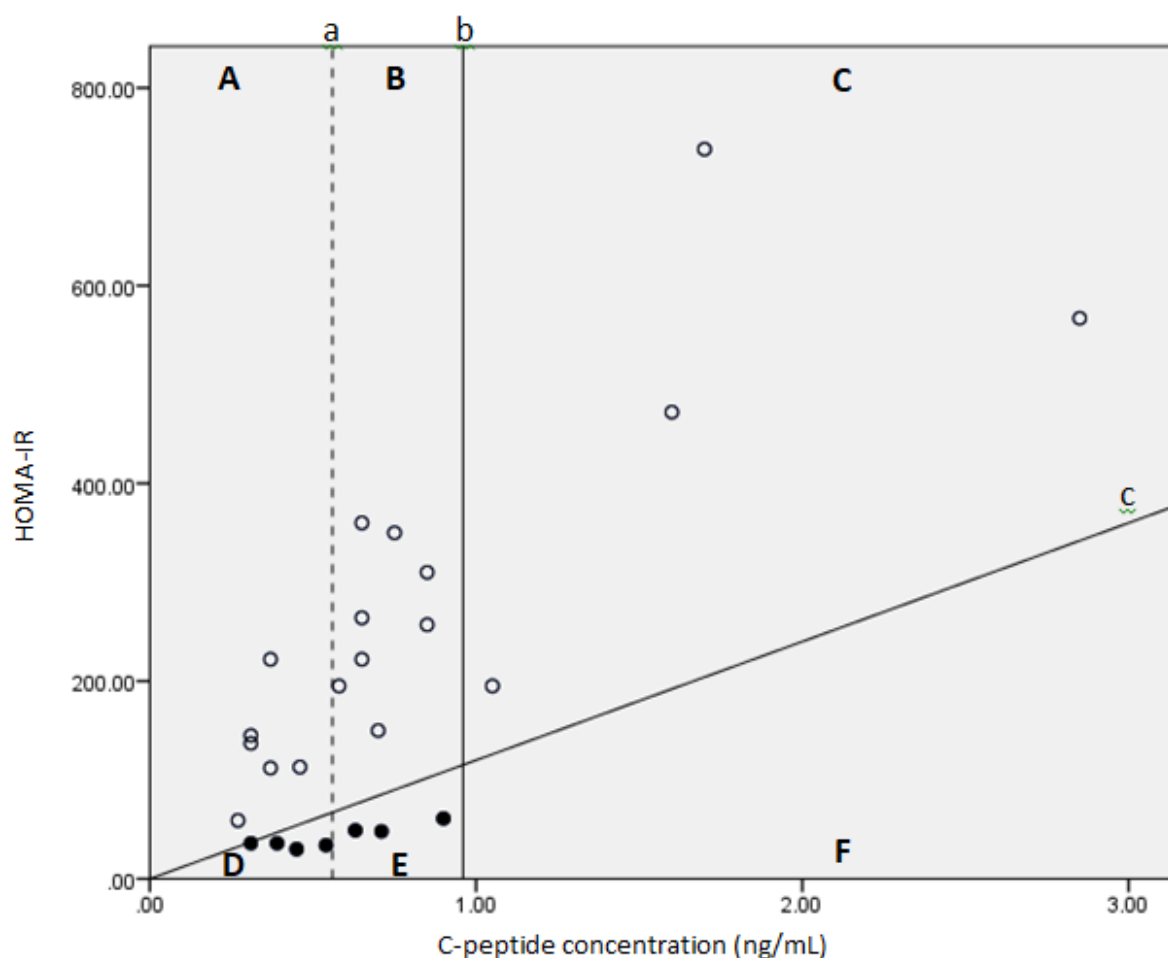


Figure 3 Scatter plots of the relationship between HOMA-IR and fasting serum C-peptide concentration (ng/mL) in each individual subject with cut-off values for categorizing the subjects were shown. Four subject categories were defined as follows: non-IR DM group (areas A and B), IR DM group or non-IR DM group with the risk of IR development (area C), normal group (area D and E), and uncategorized group (area F). N=25 (normal group; black dots; n=7, DM group; white dots; n=18)

Table 1 Statistical analyses of the grouping criteria.

Area1	Area2	Mean Difference (1-2)	Std. error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	B	-.36167	.18740	.073	-.7611	.0378
	C	-1.45167*	.22398	.000	-1.9291	-.9743
B	A	.36167	.18740	.073	-.0378	.7611
	C	-1.09000*	.21249	.000	-1.5429	-.6371
C	A	1.45167*	.22398	.000	.9743	1.9291

* The mean difference is significant at the 0.05 level

Discussion

Insulin is essential for controlling blood glucose levels in the body. DM is caused by a lack of insulin production and action. Monitoring the clinical efficiency of diabetes treatment normally relies on short-term blood sugar representatives (e.g., fasting blood glucose concentration) and long-term blood sugar representatives/glycated molecules (e.g., serum fructosamine, and serum hemoglobin A1C; HbA1C) (Sherwani *et al.*, 2016). These parameters basically reflect circulating sugar dynamics, not pancreatic beta cell function. To evaluate a pancreatic beta-cell function, secreted insulin or C-peptide is used as a direct representation (Park *et al.*, 2021). However,

continuous monitoring of insulin is not practical in diabetes cases receiving exogenous insulin administration since the ELISA kit and general detection assay cannot distinguish the difference between endogenous and exogenous insulin in blood samples. In this regard, circulating C-peptide was used as an endogenous insulin representative. C-peptide is released by islet beta cells and shares a precursor, proinsulin, with insulin during a rise in blood glucose. Subsequently, proinsulin can be degraded into one molecule of insulin and one molecule of C-peptide. As a result, the molecular weight of C-peptide is identical to that of an individual's self-secreted insulin (Behrend *et al.*, 2018). Moreover, the decomposition rate of C-peptide in the body is longer compared to the rate of

insulin (with a half-life of 20-30 minutes versus 3-5 minutes for insulin), allowing for a more consistent test window for variable beta cell responsiveness (Yosten *et al.*, 2014). C-peptide levels are useful for characterizing DM types. Low levels may indicate type 1 DM or advanced type 2, while high levels suggest insulin resistance, the early stage of type 2 DM, or insulinoma. Normal C-peptide levels typically indicate a healthy production of insulin (Venugopal *et al.*, 2018). After repairing the common calcaneal tendon, there is no difference in the maximum tendon strain between fixing and not fixing the tarsal joint, and muscle contraction continues during weight bearing (Lister *et al.*, 2009). Therefore, when common calcaneal tendon rupture occurs, a TOS method that can withstand strong tension is necessary.

In this investigation, the trend of fasting serum C-peptide concentrations in canine diabetes cases were higher than those in normal dogs, although the difference was not statistically significant. However, within the human referent range, a C-peptide level of less than 0.2 nmol/l is associated with type 1 diabetes mellitus, whereas it ranges from 0.3 to 0.6 nmol/l in healthy individuals. Because of beta cell malfunction, type 1 diabetes should have lower C-peptide levels than the normal group. This different outcome in this study may be due to the mixing of the IR population (type-2 DM), which has a high C-peptide level in the DM group (Venugopal *et al.*, 2018). However, the parameter itself could not suggest glucose homeostasis. The HOMA-IR was calculated and analyzed further, and it was shown to be significantly greater in the DM group compared to the normal. This could imply that HOMA-IR can be utilized as a surrogate parameter to differentiate between non-diabetic and diabetic dogs, which is similar to the human study, by identifying the HOMA-IR cut-off (Park *et al.*, 2021). Moreover, HOMA-IR is also used to assess insulin resistance in diabetic and non-diabetic patients in humans and provides information on beta cell function indirectly. (Hagner *et al.*, 2003; Khan *et al.*, 2018). A greater HOMA-IR measurement indicates lower insulin sensitivity and could point to insulin resistance in type 2 diabetes (Bonora *et al.*, 2000). However, the HOMA-IR approach is extensively used and practical for assessing insulin resistance, the limitations are associated with population variability and the inability to evaluate the dynamic change of insulin sensitivity (Yang *et al.*, 2021). The gold-standard assay for detecting IR is a hyperinsulinemic-euglycemic clamp, which is time- and budget-consuming (Singh and Saxena, 2010).

To establish the clinical approach for screening/detecting the presence of IR in diabetes cases, the relationship between HOMA-IR and fasting serum C-peptide concentration in each individual subject was analyzed. The cut-off values were set up. Based on the designed cut-off values and the scatter plot of the relationship between HOMA-IR and fasting serum C-peptide concentration, each individual subject could be categorized into four categories. The first group was "non-IR DM group" (areas A and B). This group showed fasting blood glucose above 120 mg/dL and fasting serum C-peptide within the mean+2SD of the normal group. Based on the evidence

that type 1 diabetes affected the majority of canine diabetics, the population of this group was greater than the other. The second one was "IR DM group" or "non-IR DM group with the risk of IR development" (area C). This group had fasting blood glucose above 120 mg/dL and fasting serum C-peptide above mean+2SD of the normal group. This could be considered type II diabetes since the increased C-peptide secretion could not control the fasting blood glucose within the normal range. To confirm IR, animals should undergo a hyperinsulinemic-euglycemic clamp or be monitored for exogenous insulin usage if more than 1 U/kg/dose fails to produce a response or 1.5 U/kg fails to reduce BG below 300 mg/dL (Behrend *et al.*, 2018). The third group was "normal group" (areas D and E). This group had fasting blood sugar and C-peptide concentrations within the normal range. The fourth group (area F) was an uncategorized group that had dramatic C-peptide value with low to normal HOMA-IR; hence "insulinoma" was suspected.

To improve the insulin sensitivity for glycemic control, an on-top metformin treatment along with the exogenous insulin administration (50% insulin dose reduction) was conducted in one pilot canine diabetes case. The results suggested a trend of beneficial effects of metformin treatment as illustrated by the decreased blood glucose and the increased C-peptide secretion. Metformin improves the responsiveness of bodily tissues to insulin, particularly muscle and adipose tissue, by increasing insulin-mediated insulin receptor tyrosine kinase activity, which activates post-receptor insulin signaling pathways. This is referred to as improving insulin sensitivity and glucose utilization (Wiernsperger and Bailey, 1999; He, 2020). The increase in C-peptide production may indicate that metformin has restored pancreatic islet function through decreasing beta-cell damage caused by persistent hyperglycemia and hypertriglyceridemia and reduced stress load on beta-cell function (Patanè *et al.*, 2000; Hur and Lee, 2015). However, the metformin preparation used in this study was a human-use 500 mg tablet. Preparation of an oral solution was necessary to address the dosing issue identified during protocol adjustment, which had resulted in gastrointestinal upset.

The limitation of the present study is its small sample size. In the DM group, risk factors for insulin resistance (IR), such as hypothyroidism, pancreatitis, hyperadrenocorticism, and steroid use, were not determined to screen for diabetic (DM) patients. There was no representation of insulinoma. Moreover, as of now, there is no standard conventional method for comparison. Increased sample size may be advantageous in future studies to generate sensitivity and specificity of this diagnostic approach.

In conclusion, the CUVET DM-IR grid I, with the criteria mentioned above, might be used as a preliminary clinical tool for screening and detecting the presence of IR in first-diagnosed and continue-treated diabetes cases. Additionally, the concept of improving insulin sensitivity might benefit glycemic control in diabetes cases. Further study with more recruited subjects is indeed necessitated to strengthen the clinical impact.

Conflicts of interest: None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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