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Untargeted metabolomic analysis in non-fasted diabetic dogs by UHPLC-HRMS

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Abstract

Introduction: We recently identified variances in serum metabolomic profiles between fasted diabetic and healthy dogs, some having similarities to those identified in human type 1 diabetes.

Objectives: Compare untargeted metabolomic profiles in the non-fasted state.

Methods: Serum from non-fasted diabetic (n=6) and healthy control (n=6) dogs were analyzed by liquid chromatography-high resolution mass spectrometry.

Results: Clear clustering of metabolites between groups were observed, with multiple perturbations identified that were similar to those previously observed in fasted diabetic dogs.

Conclusion: These findings further support the development of targeted assays capable of detecting metabolites that may be useful as biomarkers of canine diabetes.

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Author Contribution Statement

A.L.O. conceived of the study, collected the data, analyzed and interpreted the data, and wrote the manuscript; T.J.G. conducted the analytical measurements, performed statistical analysis, analyzed and interpreted the data, and wrote the manuscript. C.W. conceived of the study, analyzed and interpreted the data, contributed to the discussion, and reviewed/edited the manuscript; M.A.A. conceived of the study, contributed to discussion and reviewed/edited the manuscript.

Author Conflict of Interest Statement

All authors declare that they have no conflicts of interest.

Compliance with Ethical Standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Data Availability Statement

The metabolomics and metadata reported in this paper are available via Metabolomics Workbench www.metabolomicsworkbench.org, study identifier PR000396.

Keywords

Canine diabetes mellitus; untargeted metabolomics; type 1 diabetes; metabolites; biomarkers; ultra high performance liquid chromatography; high resolution mass spectrometry

Introduction

Canine diabetes mellitus is a common disorder with potential parallels in pathogenesis to human type 1 diabetes (T1D) (O'Kell et al., 2017b, Nelson and Reusch, 2014). Although the disease in dogs is thought complex and likely multifactorial with respect to pathogenesis (Gilor et al., 2016, O'Kell et al., 2017b), insulin deficiency appears in many cases (Fall et al., 2008, Montgomery et al., 1996). The occurrence of other components characteristic of human T1D, such as autoimmunity have, however, been inconsistently reported in dogs (recently reviewed in (O'Kell et al., 2017b, Gilor et al., 2016)).

Recently, metabolomic analysis of human diabetes has identified a variety of metabolites associated with risk for development of T1D and type 2 diabetes (T2D) (Guasch-Ferré et al., 2016, Overgaard et al., 2016), as well as with onset of overt disease (Xu et al., 2013, Dutta et al., 2016). Other than our initial pilot study (O'Kell et al., 2017a), research evaluating metabolomics in canine diabetes is unfortunately lacking. Interest in metabolomics is growing in veterinary research, and several other canine diseases have been evaluated with some promise for potential biomarker and/or disease mechanism discovery (Gookin et al., 2018, Minamoto et al., 2015, Li et al., 2015).

Thus far, methods to diagnose canine diabetes prior to symptomatic onset are not available, and earlier disease detection may be key to understanding disease pathogenesis (O'Kell et al., 2017b). Identification of serum biomarkers using metabolomics is, therefore, a potential strategy involving an initial untargeted approach within the discovery phase (i.e., a hypothesis generating approach). As an initial step in this direction, we recently identified variances in serum metabolomic profiles between fasted diabetic and healthy dogs, some having similarities to those previously identified in human T1D patients (O'Kell et al., 2017a). Given that obtaining fasted samples in diabetic dogs can be difficult in clinical practice, the purpose of the study reported herein was to expand those initial efforts to compare untargeted metabolomic profiles in non-fasted diabetic and healthy control dogs by ultra high performance liquid chromatography- high resolution mass spectrometry (UHPLC-HRMS). This, as key variances may reside in metabolic analysis between fasted and non-fasted canines.

Materials and Methods

Case Selection

Dogs with naturally occurring diabetes mellitus (n=6) and breed matched healthy control dogs (n=6) were enrolled. All dogs were recruited from the hospital population at the University of Florida (UF) Small Animal Hospital. The study was approved by the UF Institutional Animal Care and Use Committee (IACUC #201609360) and the Veterinary Hospital Research Review Committee. All owners provided written informed consent before

study enrollment. The diagnosis of diabetes was made prior to study enrollment based on compatible history and clinical signs (polyuria, polydipsia) along with persistent hyperglycemia and glucosuria. All diabetic dogs were being treated with exogenous insulin at the time of sample collection per standard of care practice. Inclusion criteria for diabetic dogs included age >1 year, body weight >5 kg, and if female, dogs must have been spayed prior to the diagnosis of diabetes. The time of blood collection in relation to feeding was recorded. Control dogs were included if they were >1 year of age with body weight >5 kg, receiving no medications other than monthly flea/tick/heartworm preventatives, and were breed-matched to diabetic dogs. Blood was collected from each control dog in a similar time frame after a meal to the breed-matched diabetic dog. For both groups, blood was collected by routine venipuncture from the jugular vein using a needle and syringe into a red top vacutainer tube containing clot activator. Serum was separated 20–30 minutes after collection and frozen immediately at -80°C .

Metabolite extraction and analysis

Metabolite extraction and analysis was performed as previously described (O’Kell et al., 2017a), using a Thermo Q-Exactive Orbitrap mass spectrometer (mass resolution set to 35,000 at m/z 200) with Dionex UHPLC and autosampler. Metabolites were aligned, gap filled, and identified using MZmine (Pluskal et al., 2010). Lipids were identified by fragmentation pathways from MS/MS, and other metabolites were matched to an internal retention time library for identification. Metabolites were identified according to MSI Level 1 guidelines. Metaboanalyst, an open source R-based program for metabolomics (www.metaboanalyst.ca), was used for further statistical analysis. Values not present in 80% of the data were removed, and missing values were imputed using k-nearest neighbor. Data was interquartile range filtered, sum normalized, log2 transformed, and autoscaled. The data was normally distributed based on visual examination of the data following sum normalization in Metaboanalyst. Autoscaling converts all standard deviations to one, and therefore variances are homogenous. The combined positive and negative ion data sets were used for statistical analysis.

Statistical analysis

A Mann Whitney U-test or Fisher’s exact test was used to compare patient characteristics (age, body weight, sex) between groups (GraphPad Prism Software v7.04). A two-sided t-test (adjusted for multiple comparisons using false discovery rate) was used to compare metabolites across the two groups, and heatmaps of significantly different features were generated. $P < 0.05$ was considered significant. Following this analysis, unknown metabolites comprising the top 50 (based on lowest p-values) were searched against the human metabolome database (HMDB) for possible matches. The search had a tolerance of 5 ppm for positive ion mode and 10 ppm for negative ion mode.

Results and Discussion

Patient Characteristics

Six diabetic and six healthy control dogs were enrolled. The diabetic group had a median age of 11.25 years (range 9–14.25 years), body weight of 10.2 kg (range 6.7–38.5 kg), and

contained 4 spayed females and 2 neutered males. The healthy control group had a median age of 5.5 years (range 2.5–11.75 years), body weight of 10.1 kg (range 5.4–49.5 kg) and contained 1 spayed female and 5 neutered males. Only age was significantly different between groups ($p=0.015$). Breed distribution in each group was Labrador retriever (2), shih tzu (1), dachshund (1), and mixed breed (2). Detailed patient characteristics are available in Supplemental Table 1.

Metabolite Results

Figure 1 displays heat maps of (a) significantly different known metabolites, and (b) the top 50 (based on lowest p -values) significantly different metabolites from both the unknown and known detected metabolites. In (b), all of the top 50 were unknown metabolites. Potential identification of unknown metabolites with available information based on HMDB are presented in Table 1. Clustering of metabolite up- and down-regulation is evident when comparing the diabetic group to the healthy group. Significant differences in known metabolites, along with major metabolic classes/pathways, as well as address of whether metabolites were up or downregulated in diabetic dogs compared to healthy dogs, are presented in Supplemental Table 2. Also displayed in this Table are metabolites that were similarly modulated in our previous study in fasted diabetic dogs (O'Kell et al., 2017a). Notable findings include downregulation of metabolites involved in tryptophan metabolism (anthranilate, kynurenine, 5-hydroxyindoleacetic acid) and downregulation of multiple amino acids in diabetic dogs compared to healthy control dogs. Several of these perturbations were also observed in the fasted dogs in our previous study (Supplemental Table 2). The microbial origin LL-2,6-diaminoheptanedioate and citramalate were downregulated and upregulated, respectively in diabetic dogs, which was also similar to our previous study. Interestingly, glucose levels were not significantly different based on the t -test, but this may have been a result of overlap in blood glucose values (possibly due to insulin therapy lowering glucose in the diabetic group) and/or type II error (Supplemental Figure 1).

Discussion

The alterations in tryptophan metabolism differ from those found in human diabetes. In a study of human T2D, kynurenine was increased in patients compared to control subjects (Oxenkrug, 2015). In patients with T1D, anthranilic acid and tryptophan were higher in diabetic patients compared to controls, but kynurenine was not different (Oxenkrug et al., 2015). However, in two rodent models of T2D, the spontaneously diabetic Torri rat and the Ostuka Long-Evans Tokushima Fatty rat, kynurenine was decreased in pre-diabetes (Yokoi et al., 2015). The effects of sex on tryptophan should be considered given that in rats (Yao et al., 2018, Deac et al., 2015) and humans (Deac et al., 2015), tryptophan as well as related metabolites were found to be higher in males. There is evidence that this difference may be related to reproductive hormones (Deac et al., 2015, Giltay et al., 2008). Although in our study the diabetic group had 4 females and 2 males compared to 5 males and 1 female in the control group, all dogs were castrated and thus endogenous circulating reproductive hormone levels should be low and static and may be less likely to be the cause of lower tryptophan metabolites in the diabetic dog group studied here. Further investigation of the

role of sex on tryptophan metabolites as well as their role as biomarkers of diabetes and pre-diabetes in dogs is warranted.

Branched chain amino acids (BCAAs) have been found to be increased in both human T1D (Dutta et al., 2016) and T2D (Xu et al., 2013). In T2D, branched chain amino acids have shown promise as biomarkers of prediabetes, insulin resistance, and future risk of future disease (Gar et al., 2018). Additionally, elevations in BCAAs have been documented before the onset of autoimmunity in children who later developed T1D, but then normalized when subjects became autoantibody positive (Oresic et al., 2008). In our study, BCAAs were not observed to differ significantly between non-fasted diabetic and control dogs based on our analysis in this study, except for an upregulation of an unknown metabolite with the possible identity of n-acetylleucine, a derivative of the BCAA leucine. This also differs from our investigation of fasted dogs in which the branched chain amino acid valine was upregulated in diabetic compared to control dog groups (O'Kell et al., 2017a). Potential reasons for this discordance include the small sample size or the effects of feeding. Although, to the authors' knowledge, the effects of feeding have not been reported using serum metabolomics in dogs, it has been demonstrated that the urine metabolome of dogs (Söder et al., 2017) and plasma metabolome of humans (Moriya et al., 2018) are affected by feeding. Our goals in the search for biomarkers of canine diabetes are to ideally identify metabolites that are not affected by feeding status to optimize the practicality of testing in a real world clinical setting. Further studies evaluating serum metabolites under fasted and fed states in canine diabetes are warranted and ongoing. Also, the effects of age on metabolomic profiles in dogs have not been investigated to date and should be considered moving forward. In humans some urine metabolites have differed between the young and old (Slupsky et al., 2007); therefore, future studies to examine the effects of age are also warranted.

One of the limitations of MS is that, due to high sensitivity/ability to measure nanomolar concentrations, many new and unknown metabolites may be detected that have not yet been identified (Emwas, 2015). Another commonly used method in metabolomics, nuclear magnetic resonance (NMR) spectroscopy, does not have the same limitation with respect to unknown metabolites, but has lower sensitivity and detects fewer metabolites in sample (Emwas, 2015). No single method provides comprehensive metabolite detection and identification, but HPLC-MS, as used in this study, is very widely used in biological sciences (Emwas, 2015). In Table 1, we have added possible identifications to unknowns from Figure 1b that had data available in HMDB. Several of the potentially identified unknown metabolites (tyrosol-4-sulfate, 3,4-methylenesuccinic acid, 2-phenylethyl butanoate, 3-methyl sulfolene, S-2-propenyl propanethioate) may be related to food ingestion. The metabolite possibly identified as dihydrolipoate was downregulated in the diabetic dog group; it is the reduced form of lipoic acid and both are anti-oxidants and additionally may increase cellular uptake of glucose (Rochette et al., 2015). Hyperglycemia causes increases in reactive oxygen species and oxidative stress, which may be involved in various chronic complications resulting from diabetes in humans (Rochette et al., 2015). Lipoic acid therapy may be beneficial in the treatment of some diabetic complications in people (Rochette et al., 2015), and may help delay cataract formation in diabetic dogs (Williams, 2017). Malate, a tricarboxylic acid cycle intermediate, is the possible identity of another unknown upregulated in the diabetic dog group. Malate is increased in the urine of

humans with T2D with progressive chronic kidney disease (Liu et al., 2018); to the authors' knowledge, no studies in T1D or canine diabetes are available.

Conclusions

In conclusion, metabolomic profiles differed significantly between diabetic and healthy non-fasted dogs, with clear clustering of metabolites between groups based on heat map analysis. Multiple metabolomic perturbations were similar to those previously found in fasted dogs, and individual metabolites may have the potential to act as biomarkers of disease. Future directions include the need for additional studies with increased sample size to confirm these findings, along with targeted metabolomics analysis of potential biomarkers to obtain quantitative metabolite measurements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Heat maps show clear clustering of metabolites between groups, representing (a) known metabolites significantly ($p < 0.05$) different between diabetic and healthy control dog groups and (b) top 50 metabolites (based on lowest p -values) from both unknown and known metabolite pool significantly ($p < 0.05$) different between groups (all of the top 50 in this instance are unknowns.) For unknowns, the first number is the mass-to-charge ratio (m/z) and the second number is the retention time. In (a), the (+) or (–) denotes if the metabolite was detected in the positive or negative ion mode respectively. In (b), the letter “n” denotes if the metabolite was detected in the negative ion mode; otherwise it was detected in the positive ion mode. Group is indicated at the top of the figure by red (diabetic, $n=6$) or green (healthy, $n=6$). Individual dog breed corresponding to column is indicated at the bottom of the figure. The color bar on the top right explains interpretation of intensity of colors in individual squares: red indicates upregulation, blue indicates downregulation; the deeper colors indicate more upregulation (red) or downregulation (blue) compared with lighter colors. Data was sum normalized, log transformed and autoscaled.

Table 1:

Possible Identities of Unknown Metabolites from Figure 1b

M/Z Ratio (mass-to-charge ratio)	Possible Identity	Class and/or Pathway	Up or down regulated in diabetic group
n_207.0508	Dihydrolipoate	Medium chain fatty acid	Down
n_217.0175	Tyrosol-4-sulfate	Organic acid and derivatives	Down
n_225.1131	3,4-methylenesecbacic acid	Branched chain fatty acid	Down
n_126.031	1-methyl-4-nitroimidazole	Nitroimidazole	Down
n_230.0127	Benzenacetamide-4-O-sulphate	Organic acid and derivatives	Down
193.1215	2-phenylethyl butanoate	Fatty acid ester	Down
135.0298	Malate	TCA cycle intermediate	Up
124.0279	3-Methyl sulfolene	Organic acid and derivatives	Up
n_129.0365	S-2-Propenyl propanethioate	Thioester	Up
147.0305	3-Oxoglutaric acid	Organic acid and derivatives	Up
n_172.098	Hexanoylglycine/N-Acetylleucine/Isovalerylalanine	Amino acids and metabolites	Up
174.1121	Hexanoylglycine/N-Acetylleucine/isovalerylalanine	Amino acids and metabolites	Up
n_287.0384	Serylvaline	Dipeptide	Up
263.0205	Homovanillic acid sulfate/Dihydrocaffeic acid 3-sulfate	Organic acid and derivatives	Up