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Expression profiling feline peripheral blood monocytes identifies a transcriptional signature associated with type two diabetes mellitus

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Highlights

- Feline blood monocytes specifically resemble human monocytes at a transcriptional level.
- Monocytes from cats with T2D exhibit a distinct expression profile to control monocytes.
- Expression profiling implicates stress and inflammatory pathways in the pathogenesis of T2D in cats

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Abstract

Diabetes mellitus is a common disease of cats and is similar to type 2 diabetes (T2D) in humans, especially with respect to the role of obesity-induced insulin resistance, glucose toxicity, decreased number of pancreatic β-cells and pancreatic amyloid deposition. Cats have thus been proposed as a valuable translational model of T2D. In humans, inflammation associated with adipose tissue is believed to be central to T2D development, and peripheral blood monocytes (PBM) are important in the inflammatory cascade which leads to insulin resistance and β-cell failure. PBM may thus provide a useful window to study the pathogenesis of diabetes mellitus in cats, however feline monocytes are poorly characterised. In this study, we used the Affymetrix Feline 1.0ST array to profile peripheral blood monocytes from 3 domestic cats with T2D and 3 cats with normal glucose tolerance. Feline monocytes were enriched for genes expressed in human monocytes, and, despite heterogeneous gene expression, we identified a T2D-associated expression signature associated with cell cycle perturbations, DNA repair and the unfolded protein response, oxidative phosphorylation and inflammatory responses. Our data provide novel insights into the feline monocyte transcriptome, and support the hypothesis that inflammatory monocytes contribute to T2D pathogenesis in cats as well as in humans.

Abbreviations

BCS Body condition score

MBA Multiple biochemical analysis

T2D Type 2 diabetes mellitus

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PBMC Peripheral blood mononuclear cells

PBM Peripheral blood monocytes

RIN RNA Integrity Number

ROS Reactive oxygen species

Keywords: feline, diabetes mellitus, peripheral blood monocytes, microarray

Introduction

Diabetes mellitus is a common endocrine disease in cats; 1 in 200 domestic cats, and 1 in 50 Burmese cats in Australia, are diabetic (Lederer et al., 2009; McCann et al., 2007). In cats the disease usually presents with polydipsia, polyuria, polyphagia, weight loss, hyperglycaemia and strong glucosuria (Reusch, 2010a). As affected cats are usually middle-aged or older, concurrent disease may occur, particularly pancreatitis, hepatic lipidosis, cholangiohepatitis and inflammatory bowel disease. Common disorders occurring secondary to feline diabetes mellitus include urinary tract infection, renal disease and peripheral neuropathy (Scott-Moncrieff, 2010).

Diabetes mellitus in cats is very similar to type 2 diabetes mellitus (T2D) in humans, especially with respect to the role of obesity-induced insulin resistance, glucose toxicity, decreased number of β-cells and pancreatic amyloid deposition (Osto et al., 2013; Zini, 2009). Risk factors associated with development of T2D in cats include obesity, inactivity, genetic predisposition (Forcada et al., 2014), breed (O'Leary et al., 2013), increasing age, being a desexed male (McCann et al., 2007), and administration of glucocorticoids or megoestrol acetate (often administered to treat inflammatory disease, cancer or diseases with immune dysregulation). However, there is a lack of understanding of the genetic factors

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and molecular mechanisms that predispose cats to developing T2D, determine the likelihood of disease progression, and contribute to the development of co-morbidities.

Human patients with T2D exhibit insulin resistance associated with obesity and an inflammatory response, with increased reactive oxygen species (ROS) generation and oxidative DNA damage resulting from hyperglycaemia (Manoel-Caetano et al., 2012). Inflammation has been suggested to be the common mechanism linking glucotoxicity, lipotoxicity and β-cell dysfunction, and also contribute directly to the development of T2D-associated complications such as retinopathy and nephropathy, and compromised tissue repair(Conde et al., 2010; Manoel-Caetano et al., 2012; McNelis and Olefsky, 2014; Meier et al., 2014). Adipose tissue in obese humans secretes chemoattractive factors which recruit monocytes and other leucocytes into adipose tissue, where they and their differentiated progeny, macrophages, secrete pro-inflammatory cytokines such as TNF, IL-6 and IL-1β that are implicated in driving the development of insulin resistance and T2D (Conde et al., 2010). Peripheral blood monocytes are thus implicated in the pathogenesis of T2D, and, because they circulate systemically and respond sensitively to environmental changes (e.g. hyperglycaemia and hyperlipidaemia), may reflect tissue damage and disease status. Several studies in T2D in humans investigating alterations in peripheral blood mononuclear cells, and monocytes in particular, support the activation of genes involved in mitochondrial oxidative phosphorylation, insulin signaling pathways, nuclear receptors, inflammatory and immune responses, responses to oxidative stress and hypoxia, fatty acid processing and DNA repair (Giulietti et al., 2007; Lackey and Olefsky, 2016; Liu et al., 2007; Manoel-Caetano et al., 2012; Takamura et al., 2007). Other studies, however have failed to detect an inflammatory signature (Baldeon et al., 2015). Further studies are clearly required to define the pathogenic role of monocytes and the inflammatory pathophysiology of T2D.

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Due to the similarities between human and feline T2D, this study aimed to improve understanding of feline T2D pathogenesis by investigating whether peripheral blood monocyte gene expression is a sensitive indicator of the diabetes microenvironment in cats. Despite knowledge of risk factors that make cats predisposed to diabetes, no early diagnostic screening tests are available, and no markers are known that predict which at-risk cats will progress to develop diabetes. Feline innate immune cells, including monocytes, are poorly characterised. Adipose tissue macrophages from obese cats, like obese humans, express IL-6 and IFNɣ, resembling the obesity induced phenotype switch of murine and human ATMs (Ampem et al., 2016). Feline monocytes can be differentiated ex vivo into potent phagocytic macrophages, in response to the growth factors Colony Stimulating Factor (CSF)-1 and Granulocyte Macrophage CSF (Gow et al., 2013; Sprague et al., 2005), and respond to inflammatory stimuli through Toll-like Receptor 4 with production of the inflammatory cytokine TNF (Tamamoto et al., 2013). However, to our knowledge the feline monocyte transcriptome has never been studied. We profiled monocyte gene expression in insulin-treated cats with T2D and controls and report a T2D-associated expression signature.

Materials and Methods

Case selection and collection of EDTA blood samples

Three diabetic and three control cats were enrolled in this study. Clients of The University of Queensland Small Animal Veterinary Teaching Hospital who owned cats believed to have well-controlled diabetes mellitus were invited to participate. Diagnosis of diabetes was based on consistent clinical signs including polydypsia, polyuria, polyphagia, hyperglycaemia and glucosuria. The latter occurs when blood glucose exceeds the renal threshold, which is approximately 14-16 mmol/L in healthy cats (Martin, 2002). Cats with diabetes mellitus in this study had no clinical or laboratory evidence of other causes of insulin resistance such as

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acromegaly, hyperadrenocorticism, or pancreatitis. Cats with acromegaly or hyperadrenocorticism typically require insulin doses in excess of 1.5 U/kg to control glycaemia and have other physical examination findings characteristic of their underlying disease (Reusch, 2010b). Of the diabetic cats enrolled in this study, one was Burmese, one a domestic medium hair, and one a Cornish Rex. Non-diabetic, control cats were Burmese and had been clinically characterised in a previous study which performed glucose tolerance testing to determine blood glucose reference intervals in healthy, client-owned cats. Casual (fasted or after feeding) blood glucose concentrations in healthy cats of a variety of ages and breeds rarely exceeded 6.5mmol/L (3 of 22 measurements) (Reeve-Johnson et al., 2012), suggesting 6.5 mmol/L is a stringent cut-point for determining normal blood glucose concentrations in client-owned cats, although published laboratory reference ranges often extend to 7 or 8 mmol/L (Reynolds et al., 2008). Control cats recruited for this study were of a similar age to diabetic cats, had a blood glucose concentration of up to 6.5 mmol/L, no history of insulin or oral hypoglycaemic therapy, and normal glucose tolerance (Reeve-Johnson et al., 2013). Ethical approval was obtained from the University of Queensland animal ethics committee (Certificate ANRFA/SVS/530/12 and SVS/284/10).

Animals were weighed and had body condition score (BCS) (laflamme, 1997) measured at the time of blood collection (5-6 mL collected from the jugular vein). Diabetic cats were fed and had insulin the morning of blood collection. Control cats were fasted the morning of blood collection. One mL of blood was preserved as serum for measurement of fructosamine, feline pancreatic lipase immunoreactivity (fPLIμ), total thyroxine (TT4) and multiple biochemistry analysis (MBA). A few drops of whole blood were used to measure glucose concentration using a handheld glucose meter calibrated for cats (Abbott AlphaTRAK). The remainder of the blood collected was preserved with EDTA as an anti-coagulant, with 120 μL used to perform a complete blood cell profile and the residual for extracting peripheral blood monocytes (PBM).

Isolation of peripheral blood monocytes

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Feline peripheral blood monocytes were isolated from 3-5 mL of EDTA blood within 6 h of collection, using the MACS Whole Blood Column Kit, MACS Human Whole Blood CD14 Microbeads and MACS separator (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's protocol. The Miltenyi kit for isolating human monocytes employs the TUK4 anti-CD14 monoclonal antibody, which cross-reacts with feline CD14 (Bienzle et al., 2003), in contrast to the commonly used MφP9 anti-CD14 clone, which did not cross-react with feline CD14 (unpublished data). Bead-isolated monocytes were stained with CD14-PE (TUK4, Miltenyi Biotec) to determine purity. Briefly, $5x10^5$ monocytes were stained with anti-CD14-PE for 20 minutes at room temperature in PBS containing 0.5% FCS and 0.01% NaN_3, washed twice in PBS and analysed using a Gallios flow cytometer and Kaluza software (Beckman Coulter). Mean monocyte yield was 0.3 x 10(9)/L (sd = 0.3) or 300 x 10(9) monocytes/mL blood.

Total RNA extraction and microarray analysis

Total RNA was extracted using the QIAGEN RNeasy Mini kit (QIAGEN, Germany) with on-column DNase digestion according to the manufacturer's protocol. Total RNA quantity and purity was evaluated using a NanoDrop spectrophotometer (A260/A280 values 1.94-2.06) prior to couriering samples to the Ramaciotti Centre UNSW for further quality control and microarray analysis. RNA Integrity Number (RIN) and ribosomal ratios were obtained using an Agilent 2100 Bioanalyzer, prior to RNA amplification and hybridisation to the Affymetrix GeneChip® Feline whole genome 1.0 ST Array. All hybridisations had consistent probeset means and AUC values ≥ 0.95 for the detection of positive over negative controls. The FelGene 1.0ST array, comprising 34,942 gene-level probe sets; a total of 792,191 probes (median 24 probes/gene) was designed in silico from genome reference sequence and Ensembl annotations and predictions on Feline genome version felCat3. Microarray data were normalised using Affymetrix Expression Console software using the RMA algorithm, and exported for further analysis in GeneSpring GX (Agilent). Data are available on the GEO website (https://www.ncbi.nlm.nih.gov/geo/), accession GSE92586.

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Microarray and Pathway Analysis

Differentially expressed probesets were identified by filtering for expressed probesets (Robust Multi-array Average (RMA)-normalised expression ≥8.8, the expression level of the Affymetrix BioB low expression control, in at least one sample), then selecting probesets that were differentially expressed between control and diabetic cats ($p<0.05$, students t-test; multiple testing correction was not applied due to the small number of samples and our goal to capture the scope of candidate differentially expressed genes in this analysis). Samples and differentially expressed probesets were clustered using the Spearman correlation with average linkage. Probesets identified as significantly up- or down-regulated in feline T2D peripheral blood monocytes were analysed for gene ontology enrichment using the Functional Annotation Clustering tool in DAVID (version 6.8, http://david.abcc.ncifcrf.gov/) with default settings. Gene Set Enrichment analysis (GSEA) was performed using GSEA v 2.1 pre-ranked analysis (to identify transcripts enriched in feline monocytes compared to curated genesets, regardless of diabetes status) as well as standard analysis (using geneset permutation) to identify genesets enriched in T2DM compared to control monocytes.

Results

Clinical and biochemical features of study subjects

There were 6 cats enrolled in this study; 3 cats with diabetes mellitus (all diagnosed more than 6 months previously) and 3 control cats. All were neutered. Clinical and laboratory results for the cats included in this study are shown in Table 1. T2D1 and T2D3 were not believed by their owners to currently show polyuria or polydipsia whereas the owner of T2D2, the only cat with a blood glucose concentration in excess of the reference interval, was not sure. T2D2 also had increased fructosamine concentration, supporting poor control of his diabetes (Hoenig et al., 2013). At the time of this study, no diabetic cats had feline pancreatitic lipase activity (fPLI) over 12 µg/dL, which is consistent with active pancreatitis

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(Forcada et al., 2008; Reusch, 2010b). All control cats had fructosamine in the reference interval, consistent with good glycaemic control over the preceding 3 weeks, with no evidence of pancreatitis in 2 controls (fPLI concentrations < 12 μg/dL). The third control cat had a fPLi value of 22 μg/dL, however the significance of this finding is unknown as azotaemia may affect fPLi values, increased fPLi values are common in apparently healthy, normal cats, and the specificity of this test for pancreatitis is 63-91%(Lidbury and Suchodolski, 2016). Minor variations in biochemical analytes, with respect to reference intervals, were present in some cats (Table 1), none of which were considered significant, except for the increased urea in all diabetic cases, with one of these also showing inadequately concentrated urine (USG 1.018). These diabetic cases may thus have had some renal dysfunction. Blood differential analysis was within reference ranges for all cats (data not shown).

Feline peripheral blood monocytes exhibit a monocyte-enriched transcriptional signature.

Feline peripheral blood monocytes were directly isolated from whole blood using anti-human CD14 magnetic beads. Purified monocytes stained with anti-human CD14 (TUK4 clone employed in cell isolation) were analysed by flow cytometry to verify antibody cross-reactivity with feline CD14, and confirm the purity of the isolated population (Figure 1A). Total RNA was hybridised to the Affymetrix Feline GeneChip 1.0 ST Array microarray. Feline mRNA expression has previously been analysed by cross-hybridisation to human microarrays, as feline genes have generally >85% similarity to their human counterparts (Collison et al., 2012; Dowling and Bienzle, 2005), and whole blood expression profiling has been performed using a custom cDNA array (Gao et al., 2013). To our knowledge, this is only the first report of feline monocyte transcriptional profiling, using a feline-specific microarray. 15,271 probesets (of which 12,471 were assigned to 7,325 unique Ensembl or NCBI transcript IDs (6,368 annotated with a Gene Symbol), 1578 were assigned to predicted protein coding

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Genscan scaffold sequences and 1,222 were completely unannotated) were detected above background. Upon unsupervised hierarchical clustering of detected probesets the 3 T2D subjects clustered together, away from the control subjects; whilst 2 of 3 control cats exhibited strikingly similar gene expression profiles (Figure 1B,C). Control #1 (the cat who had elevated fPLi (Table 1)), had a distinct expression profile, apparently with features of both the control and diabetic profiles. Principal Component Analysis confirmed Control #1 as an outlier (explaining 39.2% of the variance in the dataset), with diabetes status driving the 2nd principal component (explaining 25.6% of the variance). Overall, Control #1's monocyte expression profile was more closely correlated to the diabetic than control cats (Figure 1B).

Gene Set Enrichment analysis (GSEA) was performed to identify genes enriched in feline monocytes, regardless of diabetes status, compared to known immunologic gene signatures (Molecular Signature (MSIG) database collection 7, comprising 4,872 genesets representing immune cell states and perturbations). Human monocyte expression signatures, identified in comparison to other major leukocytes including B and T lymphocytes, dendritic cells, neutrophils and macrophages were highly enriched in feline monocytes (Table 2). The enrichment of the steady state monocyte expression signature, compared to LPS-stimulated monocytes, suggests the absence of a strong inflammatory signature in the subject cats. Interrogating the highly curated "hallmark" collection, a set of 50 genesets representing defined biological states or processes, we found that feline monocyte-expressed genes were highly enriched for targets of the transcription factor c-MYC, and a range of biological pathways, including oxidative phosphorylation, MTOR signalling, TNF and interferon signalling (Table 2).

T2D associated gene expression signature in feline peripheral blood monocytes.

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314 probesets (208 of which were assigned gene symbols and mapped to 187 unique genes) were significantly differentially expressed between cats with T2D and control cats ($p<0.05$), the majority of which (77%) were relatively over-expressed in diabetic cats (Supplementary Table 1). Several unannotated non-coding RNA transcripts were represented among the most strongly enriched probesets in both T2D and control monocytes (Supplementary Table 1). The top differentially expressed probesets with annotated Gene Symbols are listed in Table 3). Hierarchical clustering of differentially probesets illustrates the consistency in the T2D-associated transcriptional signature identified, despite expression variability among the cats (in particular Control #1), which is also reflected in the relatively low average fold changes observed in expression (Figure 2).

To gain insight into pathways that are dysregulated in feline T2D monocytes we used two approaches, both of which are limited to the subset of probesets with Gene Symbol annotations, since gene ontology classification and expression signatures available for comparison are largely based on human (or mouse) genes. First, we sought to identify pathways and gene ontology terms that were significantly over-represented among T2D-upregulated genes, compared to their representation in the human genome. Several clusters of related ontology terms (terms are clustered according to similarity in gene membership to avoid redundancy) were enriched in the T2D expression signature, including terms associated with TGFB signalling, cell division, ubiquitination and phagocytosis (Table 4). The set of annotated genes that was relatively over-expressed in control compared to T2D monocytes was too small to perform gene ontology analysis.

Secondly, we used gene set enrichment analysis (GSEA), which takes the transcriptome-wide gene list ranked by expression in T2D v control monocytes and identifies genesets associated with cell states or biological pathways that are significantly enriched in each phenotype. T2D monocytes showed enrichment of a number of curated “hallmark” genesets

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compared to control monocytes, including E2F targets and genesets associated with allograft rejection, cell cycle, DNA repair and the unfolded protein response (Table 5). Surprisingly, the most highly enriched immunologic gene signatures were associated with T lymphocyte differentiation and activation, perhaps corroborating the allograft rejection signature. A set of genes upregulated in PBMC from human T2D patients compared to healthy controls was also enriched in the feline monocyte T2D profile. Few genesets were significantly enriched in control monocytes compared to T2D monocytes. Control monocytes were, however, strongly enriched for the Interferon alpha response geneset (2.07, FDR <0.001), and a number of the enriched immunologic genesets also supported an interferon response (e.g. viral activation, data not shown).

Given Control #1 clearly clustered away from all the other samples at a transcriptome-wide level, whilst the T2D subjects clustered together (Figure 1C), we also performed geneset enrichment analysis after excluding Control #1. This analysis identified similar genesets to those identified using the whole dataset, but additionally indicated an enrichment in genesets associated with the inflammatory response, including LPS activation of monocytes and Toll-like Receptor (TLR) signaling (Table 5). Interestingly, consistent with the higher correlation between Control #1 and diabetic cats compared to control cats, when compared to Controls #2 an #3, Control #1's monocyte expression profile showed strong enrichment for similar genesets to those identified in the T2D subjects, including TNF signalling via NFKB, MTORC1 signaling and the inflammatory response (data not shown).

Discussion

We report the first transcriptomic study of cat monocytes, confirming their similarity to human monocytes, and specific signature compared to other peripheral blood leukocytes and differentiated macrophages. Monocytes and macrophages play critical roles in the

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inflammatory cascade leading to insulin resistance, tissue damage and T2D, and represent potential targets for therapy. Circulating monocyte phenotype or function may also serve as a biomarker of disease status and progression.

T2D cats exhibited a distinct expression profile, clearly clustering together at a whole transcriptome level. Statistical analyses of differentially expressed genes were, however, hampered by the fact that Control #1 exhibited an apparent inflammatory profile, which shared similarities with both control and diabetic cats. Control #1 had an elevated fLPI, suggestive of pancreatitis, however histologic lesions consistent with pancreatitis are common in apparently clinically normal, healthy cats and are not always associated with elevated fLPI (Bazelle and Watson, 2014; Gilor et al., 2016; Lidbury and Suchodolski, 2016; Oppliger et al., 2016). The significance of these findings in cats is unknown. However, clarification of the pathogeneses of these diseases, and the relationship between pancreatitis and T2D in cats could shed light on the utility of diagnostic tests and the clinical significance of various diagnostic test results in healthy and sick cats.

Despite variation in monocyte gene expression between control cats, in particular, we identified a T2D-associated gene expression signature supporting the role of similar pathways to those identified in humans, including oxidative phosphorylation, DNA repair and the unfolded protein stress response, although there was little overlap between specific differentially expressed genes and those reported in human studies. After excluding Control #1 from our analyses, we identified enrichment of additional, inflammation-associated genesets in the T2D subjects (several of which were shared with Control #1). Together, these data suggest monocyte inflammatory and stress responses are activated in feline T2D, as has been observed in humans (Manoel-Caetano et al., 2012; van der Pouw Kraan et al., 2015). The interferon signature identified in control monocytes, on the other hand, may

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indicate a role for suppressed anti-viral capacity in the etiology of diabetes, as has previously been suggested (van der Pouw Kraan et al., 2015). T2D monocytes were also enriched for a set of genes that were upregulated in PBMC from T2D patients compared to healthy controls, further supporting parallels between disease pathogenesis in the species.

Unexpectedly, pathway analysis suggested feline T2D monocytes upregulate expression of genes typically involved in T lymphocyte activation. The relevance of this is unclear, however we do not believe this is the result of contamination during monocyte purification, given the strong monocyte expression signature observed in the whole dataset. The strong enrichment of targets of the transcription factor c-MYC, an integral cell cycle regulator that is downregulated during terminal differentiation of many cell types, including monocytes(Huber et al., 2014), in feline monocytes may suggest that cat monocytes, unlike human monocytes, retain proliferative capacity. Moreover, the enrichment of cell cycle-associated genes, as well as target genes of the transcription factor E2F that has been linked to growth arrest in monocytes(Gutsch et al., 2011), suggests cell cycle dysregulation in T2D monocytes.

T2D is common in domestic cats, but the molecular mechanisms are poorly understood and there are no biomarkers to predict the development and progression of the disease. Cats have also been proposed as a valuable translational model of T2D, due to the many features shared with human T2D (Osto et al., 2013), however limited tools are available for studying feline cellular molecular biology. Serum miRNA analysis has been employed to investigate markers of diabetes and hepatic steatosis using a murine microarray (Fleischhacker et al., 2013; Weber et al., 2015), but alterations in circulating immune cells have not yet been investigated. The current study supports the utility of PBMC in investigating and describing the pathogenesis of T2D and pancreatitis in cats.

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In conclusion, this study adds to our knowledge of innate immune cells and molecular mechanisms involved in the development of diabetes, in domestic short haired cats. Although 'healthy' feline monocyte expression in different breeds and settings has not been characterised, and was variable in this pilot study, we identified a consistent T2D signature in 3 domestic cats of different breeds with different levels of glycaemic control, associated with pathways known to be perturbed in human disease. We also note that the feline transcriptome is as yet poorly annotated, which limits the power of pathway analyses. This is a valuable dataset for the field of comparative immunology which enhances our understanding of cats as a translational model, and may contribute to the development of novel biomarkers and therapeutic approaches to prolong the lives of pets.

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Figure Legends

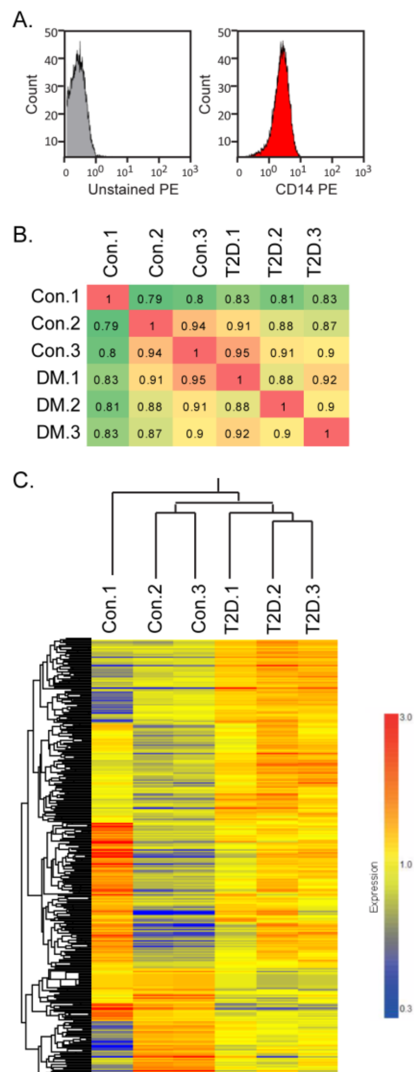

Figure 1. Feline peripheral blood monocyte expression profiling. (A) CD14 expression on purified feline peripheral blood monocytes. (B) Matrix showing Spearman correlations between control (Con) and diabetic (T2D) monocyte expression profiles. (C) Subjects and monocyte-expressed probesets (n=15,271) clustered by Spearman correlation.

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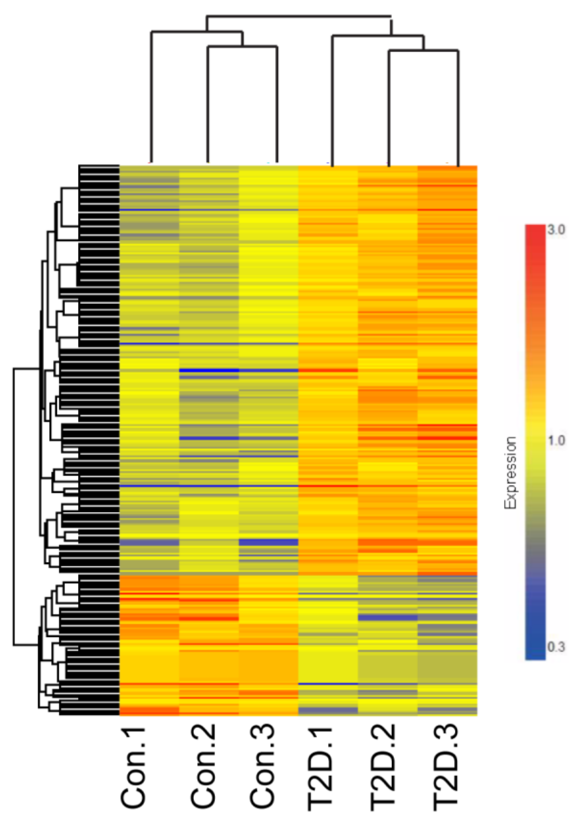

Figure 2. Differential expression in Feline T2D monocytes. Subjects and probesets (n=314) that were differentially expressed between control (Con) and diabetic (T2D) monocytes were clustered by Spearman correlation.

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Tables

Table 1: Clinical and biochemical characteristics. Values outside the reference ranges[1] indicated in parentheses, are shown in bold. P-values were generated using a 2-tailed t-test.

	Control1	**Control2**	**Control3**	**T2D1**	**T2D2**	**T2D3**	**p-value**
Breed	Burmese	Burmese	Burmese	Burmese	DMH	Cornish Rex	
Sex (all neuter)	M	F	M	M	M	M	
age (y)	12	12	12	15	13	14	0.026
wt (kg)	5.7	4.5	6.4	5.6	5.4	7.7	0.49
Body Condition Score (1-9)	6	6	7	6	5	7	0.64
SQ insulin dose (bid glargine)	no	no	no	1 U	1.5 U	1.5 U	
Blood glucose mmol/L [3.9-7.5]	6.5	6.4	5.3	6.3	**30.4**	7.6	0.33
urine glucose[2]	no	no	no	n.d.	n.d.	no	
fPLi ug/L [0.1-<3.5ug/L][3]	**22**	6.8	5.3	**10.7**	3	4.2	0.41
TT4 nmol/L (9.5-48)	31	22.9	21.5	19.7	13.9	31.8	0.61

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Fructosamine umol/L [225-265 umol/L]	243	216	221	295	604	204	0.31
urea mmol/L (5.4-10.7)	10.5	**11.9**	9.4	**15.9**	**15.5**	**18.5**	**0.007**
creatinine umol/L (70-160)	150	104	103	117	112	155	0.69
Ca mmol/L (1.75-2.5)	2.3	2.27	2.39	2.49	**2.53**	2.5	0.008
PO4 mmol/L (1.29-2.26)	0.9	**1.09**	**1.1**	**1.21**	**1.13**	1.53	0.13
total protein g/L (56-80)	80	66	72	71	**83**	**85**	**0.31**
albumin g/L (22-35)	29	**37**	**38**	**38**	**38**	35	0.48
globulins g/L (28-48)	51	29	34	33	45	**50**	**0.61**
total BR umol/L (<10)	2	<1	<1	<1	<1	<1	
ALP U/L (5-80)	32	13	15	29	64	16	0.35
ALT U/L (5-80)	52	73	**82**	**89**	**152**	65	0.29
GGT U/L (<10)	0	<1	<1	<1	<1	<1	
AST U/L (10-60)	30	40	**62**	29	36	32	0.29

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CK U/L (50-300)	140	152	**351**	**89**	124	155	0.27
Cholesterol mmol/L (1.9-3.9)	2.9	**4.5**	**6.1**	**7.1**	**5.5**	3.9	0.49
Na mmol/L (147-156)	144	155	154	155	**161**	**158**	**0.15**
K mmol/L (3.8-4.6)	4.1	4.1	4.4	3.8	**5.2**	**5.1**	**0.33**
Cl mmol/L (115-123)	110	120	122	121	122	123	0.28
HCO3 mmol/L (16-24)	21	22	22	16	21	20	0.16

[1] Unless otherwise noted reference intervals were from Vepalabs, 36 Balaclava St Woolloongabba QLD 4102

[2] Urine glucose measured by urine Multistix reagent strips (Ames) when cat had urine in the bladder at examination

[3] fPLi reference interval 0.1 - < 3.5 ug/L with active mild pancreatitis possible with concentrations over 12 (Forcada et al., 2008)

DMH: domestic medium hair

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Table 2. Geneset Enrichment analysis (GSEA) of the global feline monocyte transcriptome. NES: Normalised Enrichment Score. FDR: False Discovery Rate.

	GeneSet	GeneSet Size	NES	p-val	FDR q-val
Immunolgic Signatures	GSE29618_Up in Monocytes v Naïve B Lymphocytes	140	3.83	<0.001	<0.001
	GSE29618_Up in Monocytes v Plasmacytoid DC	142	3.62	<0.001	<0.001
	GSE29618_Up in Monocytes v Myeloid DC	139	3.59	<0.001	<0.001
	GSE22886_Up in Monocytes v Naïve T Lymphocytes	137	3.52	<0.001	<0.001
	GSE9988_Downregulated in monocytes by LPS treatment	150	3.28	<0.001	<0.001
	GSE22886_Up in Monocytes v 7-day cultured Monocytes (Macrophages)	134	3.02	<0.001	<0.001
	GSE22886_Up in Monocytes v Neutrophils	152	2.98	<0.001	<0.001
Hallmark	MYC targets	148	3.34	<0.001	<0.001
	Oxidative Phosphorylation	146	3.07	<0.001	<0.001
	MTORC1 Signaling	154	2.68	<0.001	<0.001
	TNFA Signaling via NFkB	126	2.61	<0.001	<0.001
	Interferon Alpha Response	64	2.54	<0.001	<0.001
	Unfolded Protein Response	88	2.51	<0.001	<0.001
	Interferon Gamma Response	133	2.48	<0.001	<0.001
	Protein Secretion	76	2.47	<0.001	<0.001

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	IL6/JAK/STAT3 Signaling	60	2.43	<0.001	<0.001
	PI3K/AKT/MTOR Signaling	83	2.34	<0.001	<0.001
	DNA Repair	116	2.33	<0.001	<0.001
	Adipogenesis	147	2.26	<0.001	<0.001
	P53 Pathway	141	2.25	<0.001	<0.001
	TGFB Signaling	36	2.17	<0.001	<0.001
	Apoptosis	107	2.13	<0.001	5.9E-05
	Reactive Oxygen Species Pathway	36	2.09	<0.001	1.1E-04
	Peroxisome	77	2.08	<0.001	1.1E-04

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Table 3: Top 10 significantly overexpressed probesets assigned to Gene Symbols in T2D and control monocytes.

Probeset ID	Gene Symbol	mRNA Accession	Fold Change Control/T2D
14620769	SH2D1A	ENSFCAT00000008356	0.071
14640264	ALAS2	ENSFCAT00000000908	0.284
14535457	FXC1	ENSFCAT00000004633	0.372
14616718	GPR125	ENSFCAT00000001550	0.418
14598371	SLC9A9	ENSFCAT00000009301	0.520
14615888	PLCG1	ENSFCAT00000009035	0.529
14571381	UGGT1	ENSFCAT00000001265	0.535
14616616	PHF5A	ENSFCAT00000003676	0.543
14534676	TXNDC16	ENSFCAT00000000462	0.544
14660847	TSPAN18	ENSFCAT00000004187	0.553
14594121	LSM6	ENSFCAT00000003047	1.466
14536249	COG3	ENSFCAT00000004871	1.480
14561018	TPM4	ENSFCAT00000009001	1.481
14645967	VSIG4	ENSFCAT00000007041	1.658
14637470	LRP8	ENSFCAT00000005933	1.694
14579682	P2RY1	ENSFCAT00000013021	1.743
14627820	MAP3K8	ENSFCAT00000004397	1.874
14524268	LRP6	ENSFCAT00000000044	2.231
14573976	HPSE	ENSFCAT00000018807	2.240
14557961	CCL3L	NM_001163657	2.618

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Table 4: Gene Ontology Enrichment Analysis. Functional clusters enriched among T2D upregulated genes. The enrichment score, calculated as the –log of the geometric mean of the enrichment p-values of the classification terms making up each cluster, is used to rank biological significance.

Functional Cluster (Representative Term/s)	Enrichment Score (Significance)	Term Fold Enrichment (Range)
TGFB signaling, negative regulation	1.8	2.0-9.2
Cytoskeleton, mititotic cell division	1.75	2.4-5.1
Ubiquitin conjugation pathway	1.69	1.2-9.8
Golgi apparatus	1.64	1.4-2.5
Endosome, Lysosome	1.56	2.5-6.0
Fc-gamma receptor signaling, phagocytosis	1.44	1.4-8.0

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Table 5. Geneset Enrichment in T2D feline monocytes. Selected genesets from MSIG hallmark, immunologic and KEGG pathway collections enriched in T2D compared to control monocytes.

			All Samples			Excluding Control #1		
	GeneSet	Size	NES	P-VALUE	FDR	NES	P-VALUE	FDR
Hallmark	E2F Target Genes	147	2.17	<0.001	<0.001	1.75	<0.001	0.022
	Allograft Rejection	128	2.14	<0.001	<0.001	1.28	<0.001	0.023
	Cell Cycle: G2M Checkpoint	144	1.75	<0.001	0.010	1.58	0.003	0.032
	Oxidative Phosphorylation	146	1.57	0.044	0.064	1.57	0.001	0.031
	Cell Cycle: Mitotic Spindle	156	1.42	0.007	0.196	1.40	0.017	0.095
	DNA Repair	116	1.40	0.006	0.182	1.00	0.462	0.602
	Unfolded Protein Response	88	1.35	0.050	0.247	1.51	0.015	0.035
	TNF signaling via NFKB	126	-	-	-	2.20	<0.001	<0.001
	MTORC1 Signaling	154	-	-	-	1.75	<0.001	0.010
	TGFB Signaling	36	-	-	-	1.56	0.023	0.032
	Inflammatory Response	139	-	-	-	1.35	0.027	0.121
Immunologic Signatures	GSE22886_Up in Naïve T Cells v Monocytes	146	2.15	<0.001	<0.001	1.77	<0.001	0.005
	GSE7509_Upregulated in DC by FCGRIIB stimulation	120	2.13	<0.001	<0.001	1.85	<0.001	0.002
	GSE9006_Upregulated in T2D v Healthy PBMC at Diagnosis	129	1.39	0.027	0.074	1.46	0.019	0.099
	GSE9988_Upregulated by LPS in Monocytes	115	-	-	-	1.95	<0.001	0.000
Kegg Pathways	Primary Immunodeficiency	26	2.13	<0.001	<0.001	1.93	<0.001	0.017
	Cell Cycle	85	1.97	<0.001	0.011	1.65	0.003	0.152
	T Cell Receptor Signaling	82	1.90	<0.001	0.015	1.75	0.001	0.124
	NK Cell Mediated Cytotoxicity	69	1.79	0.002	0.038	1.72	0.001	0.085
	Ubiquitin-mediated Proteolysis	101	1.77	<0.001	0.039	1.29	0.067	0.516
	Toll Like Receptor Signaling	65	-	-	-	1.50	0.021	0.384