Diabetic Complications Consortium

Application Title: Metabolomic and transcriptomic markers of diabetic changes in baboon kidney.

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1. Project Accomplishments:

The overall goal of the project was to perform profiling of metabolites and small RNAs in blood, urine, and kidney biopsies from spontaneously diabetic and healthy female baboons, using opportunistic samples collected at necropsy after euthanasia for routine colony management. The motivation was to obtain preliminary data on potential novel biomarkers of both diabetes and incipient kidney damage.

Banked samples were available for 12 diabetic female baboons (relatively few males have been collected to date; we chose to focus on females because we lacked statistical power to account for sex differences). 12 non-diabetic, non-obese controls were selected over a range of ages as close as possible to that of the animals with diabetes. Because of difficulties in collecting urine at necropsy, usable urine samples were available for 4 diabetic and 4 control animals.

Table 1. Characteristics of animals and serum samples (12 control, 12 diabetic)
Data presented as median (range)

Status	*Age, y*	*Weight, kg*	*HbA1c, %*	*Creatinine, mg/dL*	*Albumin, g/dL*	*BUN, mg/dL*
Control	13 (10-20)	17.2 (12.8-24.9)	4.0 (3.6-4.5)	1.0 (0.9-1.2)	4.0 (3.5-4.8)	15 (5-22)
Diabetic	18 (10-26)	18.9 (14.4-29.6)	11.0 (6.2-14.0)	0.8 (0.6-1.2)	3.8 (3.1-4.1)	7.5 (4-27)

Serum creatinine and blood urea nitrogen (BUN) were *lower* on average in these diabetic animals than in controls (even when corrected for age, data not shown). A similar reduction of circulating creatinine in human patients with diabetes has been reported by Harita et al. (2009), who suggested as possible cause muscle wasting leading to reduced muscle turnover. We see some evidence of increased variance in age-corrected creatinine levels in diabetic samples and will examine the effect of age of onset/duration of diabetes at death (still being confirmed from veterinary records for some animals).

2. Specific Aims:

Specific Aim 1 (SA1): Transcriptomic profiling of miRNA and metabolomic profiling of kidney biopsies from diabetic vs healthy baboons.

Specific Aim 2 (SA2): miRNA and metabolomic profiling of serum and urine from diabetic vs healthy baboons.

Results for both Aims are presented by molecular target (small RNA sequencing or metabolomic profiling).

RNA sequencing (RNAseq) of small RNAs, including exosome-bound and free microRNA (miRNA) as well as possible precursor or degradation products, was performed on kidney biopsy (***SA1***) and serum (***SA2***) samples. The kidney wedge samples were segmented into cortical and medullary fractions prior to RNA extraction. Exosomes were extracted from urine samples for RNAseq of exosomal RNA. This differential approach was based on an expectation from the literature that non-exosomal RNA in urine is likely to be products of degradation, while some free small RNA in blood and kidney may be biologically relevant. RNAseq was performed using the Illumina GAIIX Genome Analyzer platform, and sequence reads were cleaned and annotated using miRDeep. As a first analysis, the preliminary results reported here are based on 2,947 known miRNA detected in at least one sample type. In addition, we found 595 predicted novel miRNA with a miRDeep score ≥ 4.

Normalized values for known miRNA were tested for differential expression between healthy and diabetic baboons by *t*-test in R (Table 1). In general, there was little overlap across sample types; exceptions are hsa-miR-221-5p, differentially expressed at $p<0.05$ in serum and medulla, and hsa-miR-139-3p, differentially expressed in both kidney compartments. At $p<0.05$, only 1 miRNA was differentially expressed in urine, 20 in serum, 59 in cortex, and 63 in medulla. The strong evidence of differential expression with diabetes in kidney is noteworthy since no baboon had evidence of advanced nephropathy as assessed by serum creatinine. Thus, these molecules are potentially informative of preclinical responses of the kidney to diabetes.

Table 1. Leading differentially expressed miRNA. p-values <0.05 shown in **bold.** Only one miRNA differentially expressed at $p<0.05$ was found in urine; the 5 lowest p-values are given for each of the other sample types.

miRNA	***Serum***	***Urine***	***Kidney cortex***	***Kidney medulla***
hsa-let-7b-5p	**0.0136**	0.8051	0.1064	0.2395
hsa-miR-676-3p	**0.0220**	0.3610	0.9682	0.2708
hsa-miR-221-5p	**0.0290**	0.5901	0.0758	**0.0497**
hsa-miR-152-3p	**0.0338**	0.7212	0.5018	0.8907
hsa-miR-539-3p	**0.0362**	na	0.2215	0.9982
hsa-miR-335-5p	0.1653	**0.0119**	0.3420	0.0677
hsa-miR-139-3p	0.7765	na	**0.0003**	**0.0369**
hsa-miR-138-2-3p	0.3434	na	**0.0014**	0.8648
hsa-miR-195-3p	0.6196	0.3024	**0.0029**	0.5488
hsa-miR-99a-3p	na	na	**0.0035**	0.5550
hsa-miR-376c-3p	0.6616	0.3599	**0.0044**	0.6404
hsa-miR-10a-3p	0.5438	0.2977	0.1049	**0.0001**
hsa-miR-574-3p	0.1426	0.4692	0.9862	**0.0003**
hsa-miR-190a-5p	0.9428	0.4114	0.4189	**0.0014**
hsa-miR-628-3p	0.2782	0.4510	0.7189	**0.0020**
hsa-miR-125a-5p	0.1711	0.8357	0.6118	**0.0023**

At least two of the miRNA in Table 1 (let-7b-5p and miR-195-3p) have been reported as differentially expressed in mouse models of Type 2 diabetes and diabetic nephropathy (Wu et al., 2014).

Metabolomic profiling was conducted via two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GCxGC-ToF-MS) using an Agilent 7890 B gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) with Pegasus 4D ToF-MS instrument (LECO Corp., San Jose, CA, USA). Data were cleaned and aligned using ChromaToF v. 4.50.8.0. Metabolites were identified by comparing fragmentation patterns available in the NIST Mass Spectral Reference Library (NIST11/2011). Statistical analyses were performed in R; pathway enrichment analysis was performed using MetaboAnalyst (www.Metaboanalyst.ca).

SA1: *Metabolomic analysis of cortical and medullary kidney samples* is work in progress.

SA2: *Metabolomic analysis of serum and urine.* In serum samples a total of 114 metabolites were quantified, of which 88 were shared between control and diabetic animals; 11 and 17 were unique to either diabetics or controls**.** Metabolites detectable only in healthy baboons include cysteine, ribose, and malic acid, while those in the diabetic animals included antioxidants (alpha-tocopherol, ascorbic acid, linolenic acid, linoleic acid), sugar alcohols (myo-inositol, glycerol), butanoic and galacturonic acids.

In urine samples, a total of 258 metabolites were quantified of which 211 were shared between control and diabetic animals. 23 and 24 metabolites were unique to control and diabetic animal serum, respectively. Metabolites in healthy baboons include cysteine, aspartate, indole-2-carboxylic acid, purines, 2-hydroxybutanoic acid, 34-hydroxyphenylacetic acid, while those in the diabetic animals included galactose, threonine, tartaric acid, threonic acid, m-trimethylhippurate among others.

Differential metabolite accumulation was evident across sample types. In serum, the diabetic baboons showed significantly increased ($p<0.05$) sugars (maltose, fructose), amino acids (asparagine, aspartate, proline), and decreased beta-hydroxybutyric acid when compared to controls the healthy baboons (Figure 1). In urine, metabolites showing significant decreases in diabetic animals included uric acid, valerylglycine, hypotaurine, glycine, N-2-methyl-1-oxo-2-butenyl-glycine, isocitric acid, hypotaurine and fatty acids (methyl-propanedioic acid, octanedioic acid); diabetic urine samples showed increases in specific amino acids, organic acids, pinitol, uracil, and 2-deoxy-D-arabinohexonic acid.

Figure 1. Metabolic changes in serum (panels A, B) and urine (panel C).

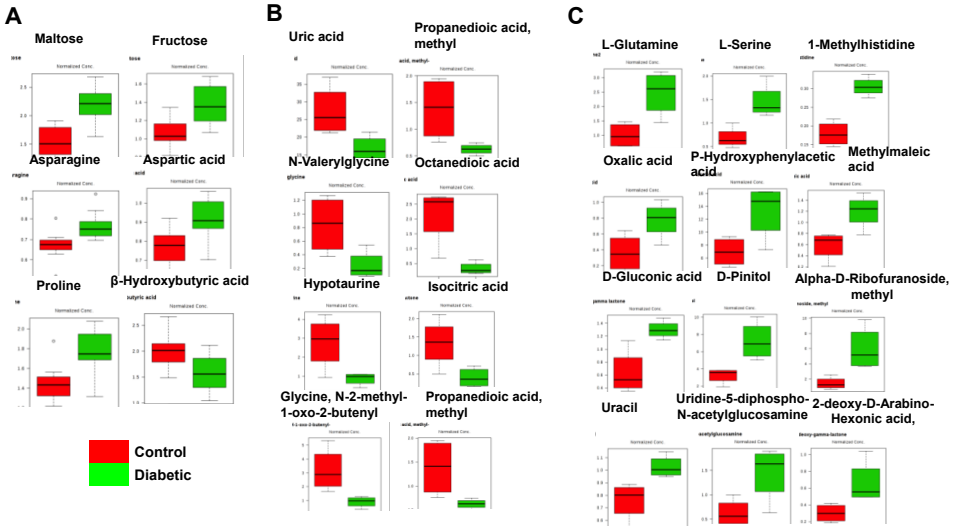
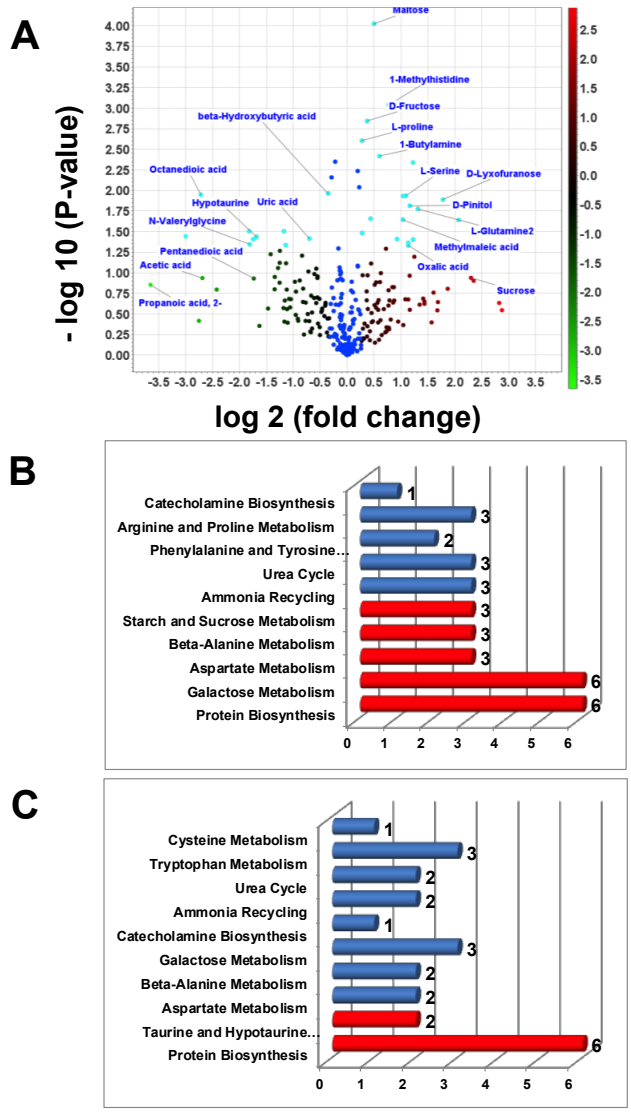
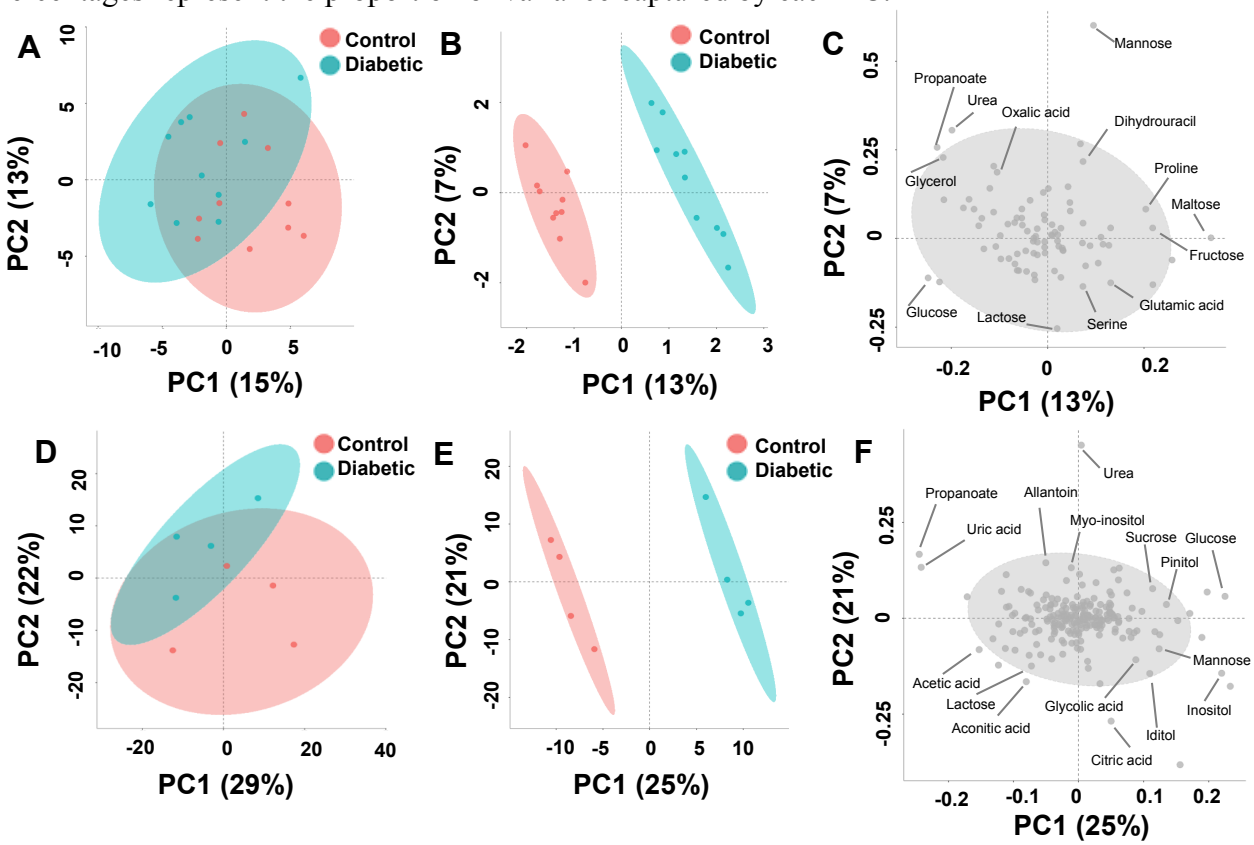


Figure 2. Panel A, volcano plot of differential accumulation of metabolites in combined serum and urine datasets. **Panel B**, pathway enrichment analysis of differentially expressed serum metabolites; **Panel C**, pathway analysis for urine. In B and C, blue bars indicate enrichment at $p<0.05$; red, $p<0.1$. Numbers at right of bars are the number of metabolites in each pathway.



A primary motivation for this pilot study was to identify potential circulating biomarkers of diabetes and its complications. Unsupervised principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed on differentially expressed metabolites to test their potential to discriminate diabetic animals from controls (Figure 3). In particular, the first two PLS-DA components provided very clear discrimination between diabetic and control animals.

Figure 3. Discriminant analysis. Top row, serum: **Panel A**, PCA; **B**, PLS-DA; **C**, PLS-DA first component loadings on specific metabolites. Bottom row, same panel sequence for urine. Percentages represent the proportion of variance captured by each PC.



References:

Harita N et al. Diabetes Care 2009; 32:424-426.

Karere G et al. BMC Genomics 2012; 13:320.

Wu H et al. Journal of Diabetes Research 2014; Article ID 920134.

3. Publications:

Biswapriya B. Misra, Ram P. Upadhayay, Vicki L. Mattern, Laura A. Cox, Anthony G. Comuzzie, Michael Olivier, Jack W. Kent Jr. Serum and urinary responses in diabetic vs. healthy baboons.

a. Manuscript in preparation.
b. Abstract accepted for oral presentation by Dr. Misra at annual meeting of the Texas Genetics Society in College Station, Texas, April 28, 2017.