Specific Aims

The prevailing theory to explain the complications of type 1 diabetes is based upon the idea that exposure to excess glucose will set off a cascade of deleterious events, initially driven by over-production of mitochondrial superoxide anions. Our data using cell culture, animal models and human subjects strongly favors a completely different hypothesis. We hypothesize that mitochondrial function and mitochondrial superoxide anion production is shut down in response to exposure to excess glucose. This effect, termed the Crabtree effect, if persistent will lead to Warburg metabolism which is closely tied to production of disease-promoting pro-fibrotic and inflammatory molecules.

The proposed studies will test this hypothesis in patients with type 1 diabetes at the metabolomic and epigenetic level and in experimental systems to prove our hypothesis and establish the molecular mechanisms driving this course of events in the diabetic kidney. Metabolomic and epigenetic markers of reduced mitochondrial function and biogenesis will be performed in urine samples from 2000 patients in the FinnDiane and CRIC cohorts. Reduced mitochondrial function will set off an inflammatory and pro-fibrotic cascade leading to progressive cell dysfunction and reduction in the glomerular filtration rate. We postulate that the pathogenetic mechanism mediating the shutdown of mitochondria in diabetes is via inhibition of PGC1α. The role of this pathway will be tested in mouse models of diabetic kidney disease using novel imaging tools and cell specific deletion of PGC1α.

Aim 1. Human urine metabolomics. Urine metabolomic profiles in patients with type 1 and type 2 diabetes will be correlated with decline in kidney function. In preliminary work, we have identified a panel of urine metabolites that reflect abnormal mitochondrial metabolism and are reduced in individuals with diabetic kidney disease. Urine samples from 1000 patients with type 1 diabetes in the FinnDiane study and 1000 patients with type 2 diabetes in the CRIC study will be assessed. The metabolomic profile will be correlated with future decline in kidney function over 6 years of follow-up. We will test the hypothesis that urine metabolite levels are an indication of renal cell dysfunction among individuals with diabetic chronic kidney disease and will be associated with the rate of decline of kidney function, independent of baseline eGFR, urine albumin to creatinine ratio, or other established risk factors for longitudinal decline in eGFR.

Aim 2. Human urine epigenetics. Urine epigenetic profiles in patients with type 1 diabetes from FinnDiane patients will be analyzed for potential mediators of reduced mitochondrial structure. Specifically we will focus on methylation of the PGC1α promoter that will lead to inhibition of mitogenesis.

Aim 3. Experimental animals: Superoxide imaging, mitochondrial function, metabolomics and epigenomics. We will employ a series of animal studies to demonstrate the relationship between mitochondrial structure and function in progressive and non-progressive models of diabetic kidney disease in mice (Akita Bl6J and F1 cross of Bl6 and DBA/2). A series of imaging and biochemical studies will determine mitochondrial superoxide anion and mitochondrial function in early and established diabetes. Using PGC1α floxed mice we will determine the causal role of PGC1α inhibition on glomerular mitogenesis and in the initiation and progression of diabetic kidney disease. Whole tissue metabolomics and whole genome methylation studies in kidney tissue and urine during the development of diabetic kidney disease will establish the relation between urine markers and renal disease. Using a systems biology approach we will then demonstrate novel biomarkers that closely reflect and track renal mitochondrial status at the metabolomic and epigenetic level.

Research Strategy –

a) Significance and Impact: The understanding of the basic mechanism of cell dysfunction in response to diabetes is central to developing effective methods to prevent, diagnose, monitor and treat the complications of diabetes. Our data from animal studies and humans challenges the dogma that elevated glucose levels leads to excess mitochondrial activity and excess production of mitochondrial superoxide anions. Our new hypothesis is that elevated blood glucose levels leads to an inhibition of mitochondrial function and synthesis and subsequent inflammation and fibrosis. If true, this characteristic response should be able to be documented in patients and animal models of diabetic kidney disease. We further hypothesize that the degree of inhibition of mitochondrial function and mitogenesis will be the basis for future impaired reduction of glomerular filtration rate, a key function of the kidney cyto-architecture. This proposal focuses on proving this concept in patients with type 1 and type 2 diabetes and progressive kidney disease and in animal models of type 1 diabetes. By demonstrating the validity of this pathway and assessing new tools to monitor this pathway in humans, the data will significantly advance our understanding of diabetic complications and alter the course of pharmaceutical approaches to treat diabetic complications.

Project Description: Mitochondria are at the heart of the cellular decision tree for energy sensing and have adapted to the constant changes in the availability of nutrients in the environment. Mitochondria are also central players in the determination of cell death and survival and for tuning metabolism in tissue-specific ways for optimum organ function. Whereas the response of mitochondria to states of energy deficits are well understood the response of the mitochondria and the cell to caloric excess is unclear. As proposed by Brownlee, the accepted theory to explain why humans develop diabetic complications is that excess calories are processed via mitochondria resulting in excess production of superoxide anion radical via the electron transfer chain [1]. The resulting accumulation of superoxide within the mitochondria would then mediate downstream pathways related to cell dysfunction. However, our exciting new data generated from animal imaging demonstrates a completely opposite set of conclusions! There is actually a dramatic reduction of intracellular superoxide in response to hyperglycemia in the kidney following onset of diabetes. Our project will test the hypothesis that reduced mitochondrial function in patients with type1 and type 2 diabetes and kidney disease predicts the rate of decline in renal function, that epigenetic changes of PGC1α promoter methylation is increased in patients with type 1 diabetes and kidney disease, and that these changes are reflective of underlying renal function in mouse models of diabetic kidney disease.

Investigators Qualifications: The proposal will bring together experts from mitochondrial biology, animal imaging, metabolomics, epigenetics, systems biology with seasoned basic science, translational, clinical epidemiologic experts in kidney disease of diabetes. The multi-disciplinary cross-talk among experts in the respective fields composing the innovative investigative team will allow for novel advances and minimize bias in adhering to long-held dogmas. Several new technologies will be brought to bear to analyze the clinical and experimental models. The application of novel quantitative metabolomic profiling and mitochondrial function will take advantage of the unique expertise of metabolic diseases at UCSD (Barshop/Naviaux/Murphy). The application of innovative techniques and equipment to assess the specific epigenetic marks (O'Connor, Ren) in our well-characterized patient populations could well develop into new clinical diagnostic and monitoring tests. Novel imaging tools in models of diabetic kidney disease (Dugan, Sharma) to query the in vivo monitoring of mitochondrial superoxide anion will be a valuable tool for research in many conditions. The outstanding expertise of our team with clinical samples and clinical studies in patients with kidney disease (Feldman, Groop, Ix, Sharma) along with systems biology (Ideker) will allow for rapid insights into the clinical significance of the data generation. Dr. Sharma will work with the investigators to insure that there is a smooth flow of experimental work that synergizes with all components of the group. The coordination of assays and organization of information flow will be directed by Dr. Sharma.

Suitability for T1D-Impact award program: The stated goals of the DP3 initiative are to encourage an application that is meant to challenge an existing paradigm of an important topic and to transform the field. Another important criterion of the DP3 is to encourage risk taking and to promote a multi-disciplinary approach. This DP3 came about an opportune time for our group. We have been critically examining the accepted theory of diabetic complications using a novel in vivo approach to chart the time course of superoxide production in diabetic complications. However, our data was diametrically opposite to what was expected based on the

standard Brownlee paradigm. There was a reduction in superoxide production despite evidence of early injury to the diabetic kidney (glomerular hypertrophy and albuminuria). Thus the data demonstrated provocative evidence that mitochondrial superoxide production was suppressed in the diabetic mice and may not play a major role in complications. However there was no prevailing theory to explain the data. Our group has developed a new evolutionary based and physiologic theory to understand the data generated and to predict downstream consequences related to the development of inflammation and fibrosis. A multi-disciplinary approach is essential as we seek to understand the individual and global response of the organism to glucose excess in humans and animal models. Our proposal breaks with the prevailing and orthodox view that mitochondrial superoxide anions are the direct cause of cell and organ dysfunction in diabetes. Our studies are directly relevant to novel biomarkers for diabetic kidney disease as we will apply novel methods and expertise to understand the urine metabolome and epigenome in humans with type 1 diabetes. The combination of expertise and proposed studies would not be able to be completed within the confines of a typical R01 mechanism.

Preliminary data (Mouse studies) Male C57BL6 mice made diabetic by multiple low-dose injection of steptozotocin (STZ) showed significantly elevated plasma glucose concentrations 4 weeks after onset of hyperglycemia. Superoxide production in kidney from live diabetic versus control mice was then measured using systemic administration of dihydroethidium (DHE) to detect superoxide and a whole animal fluorescence imaging system (OptixTM) (Figure 1). To identify the position of the kidneys in the intact animal, control and diabetic mice were co-injected with FITC-inulin 30 minutes prior to being placed in the scanner. FITC-inulin is filtered by the kidney, allowing the location of the kidneys to be established by visualizing the FITC fluorescence. DHE oxidation was prominent in normal kidneys, but surprisingly, diabetic kidneys had reduced DHE oxidation, indicating reduced superoxide production. Similar findings were noted in Akita mice from an F1 cross between Bl6 and DBA.

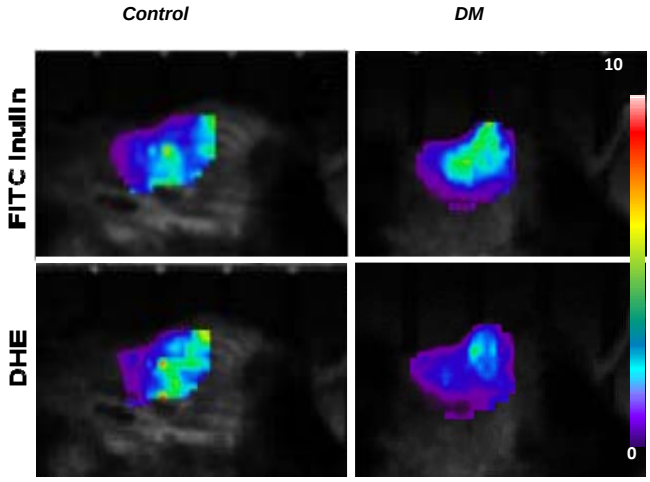

Figure 1. Imaging of kidney superoxide production in normal and diabetic mice. Live animal imaging of kidney through the intact skin in a prone, isofluorane-anesthetized mouse. Kidneys were first localized using the FITC channel, (for FITC-inulin) and then the filter settings were changed to the ox-DHE channel (Ex 470 nm, EM >590 nm) to image superoxide production in control and STZ-induced diabetic mice (DM). Fluorescence is shown using a linear pseudocolor scale (images representative of n = 6 mice per group).

As diabetes was characterized by reduced mitochondrial superoxide anion production, we examined parameters of mitochondrial structure and function in the normal and diabetic kidney. We hypothesized that an overall reduction in mitochondrial superoxide production could be due to a redistribution of carbons and electrons away from mitochondria and to the cytosol. The mitochondrial complex activity tended to be reduced in the diabetic kidney (Fig. 2 A-D). Pyruvate dehydrogenase complex (PDH) is the major gateway for pyruvate carbons to enter the Krebs cycle in mitochondria [2]. When the E1□ subunit of PDH is phosphorylated on serine 293 (PDHE1□-pSer^{293}), the enzyme is inhibited. We found that PDH was indeed hyperphosphorylated (inhibited) in the kidneys of diabetic mice (Fig. 2E). We developed a novel, real-time quantitative PCR assay for the most commonly found mtDNA deletion in mice as a marker of mtDNA damage. We found a striking, 3-fold increase in mtDNA deletions in the kidneys of diabetic mice (Fig. 2 F). All parameters of reduced mitochondrial structure and function were restored with AMPK activation of diabetic mice (Figure 2).

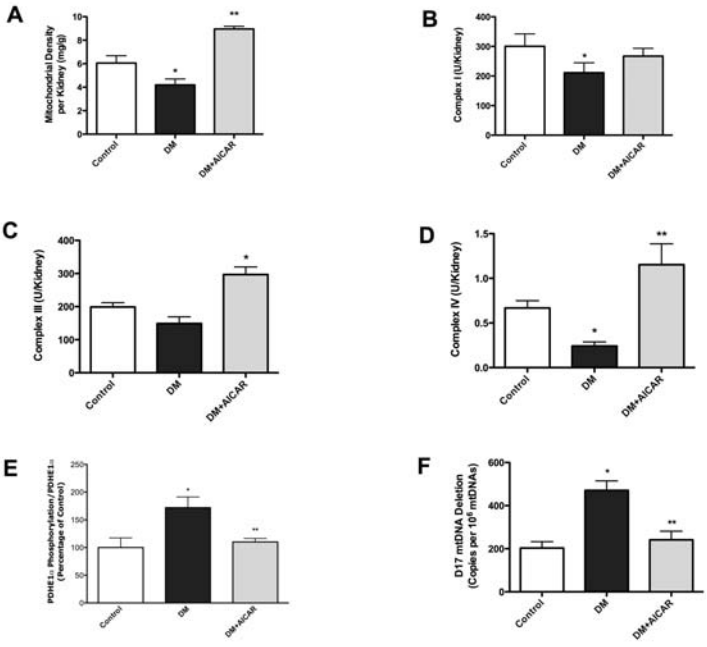

Figure 2. Diabetes-induced regulation of mitochondrial respiratory chain function, mtDNA deletion, PDH phosphorylation are reversed with AMPK activation. (A), Total mitochondrial content per gm kidney protein was measured in the control, diabetic, and diabetic mice treated with AICAR. **(B),** Complex I activity (**C),** Complex III activity (**D),** Complex IV activity measured in control, diabetic, and diabetic mice treated with AICAR. **(E),** Quantitative analysis of PDH phosphorylation in control, diabetic, and diabetic mice treated with AICAR. **(F),** D17 mtDNA deletion in kidney DNA from the groups indicated. (n≥6 per group, *p<0.05 vs control, **p<0.05 vs corresponding diabetic group).

Preliminary data (Humans) To determine if a similar pattern of reduced mitochondrial function may be manifest in patients with diabetes and kidney disease we evaluated the urine metabolomic profile in two cohorts of patients with diabetic kidney disease. The UCSD Biochemical Genetics Core is one of the first Metabolomics Units developed in the United States. Initially established by Bill Nyhan, the facility has optimized quantitative assays for many metabolites that identify mitochondrial dysfunction in patients with inborn errors of metabolism. As we hypothesized that diabetic kidney disease is an acquired form of mitochondrial dysfunction we postulated that there will be a urine metabolomic profile that will indicate mitochondrial dysfunction. We therefore performed analysis of patients to identify the metabolomic profile in patients with diabetic kidney disease. Thus far, our screening population includes 24 patients with a diagnosis of diabetic kidney disease with CKD Stage 3-4 (Mean GFR 31.79±7.329). This population was compared to a control group of 23 healthy volunteers with no diabetes or kidney disease. 24 hour urine was collected from both control and DN subjects. 120 different organic acids were quantified in each sample and the results were standardized to creatinine (expressed as mmol/mol creatinine). We compared the two groups for each of the metabolites and found 11 significant metabolites using the unpaired t test. We chose a cut off p value of P <0.00846 to have false detection rate less than 0.05 to account for multiple testing. We found that glycolic, 3–OH-isobutyric, 3-OH-isovaleric, aconitic, homovanillic, succinic, azelaic, citric acid, uracil and glutaric acid were significantly decreased in the diabetic nephropathy group. 5-OH-hexanoic acid was the only metabolite that was significantly increased in the diabetic nephropathy group. Using a second cohort of 49 patients with both type 1 (n=10) and type 2 diabetes (n=39) and CKD (Mean GFR 37.8 ± 6.45).we validated that 8 of these metabolites remained significantly different from the control population (see table 1 below). A network analysis was constructed with Cytoscape and various metabolite databases, including Kegg and UCSD generated metabolic maps (Figure 3).

	Control N=23		**Cohort1 N=24**		**Cohort 2 N=49**	
	Mean	SD	Mean	SD	Mean	SD
GLYCOLIC	34.6	15.281	10.64	3.564	11.67	5.459
n3OH_ISOBUTYRIC	27.1	17.290	12.35	6.071	12.16	8.327
n2OH_ISOVALERIC	0.69	0.558	0.42	0.646	3.10	2.583
HOMOVANILLIC	2.65	1.300	1.42	0.513	1.02	0.721
SUCCINIC*	6.08	3.489	2.85	2.684	6.61	9.395
AZELAIC	3.08	1.880	1.21	0.974	0.51	0.710
CITRIC	416.82	267.683	148.14	78.798	138.14	142.376
URACIL	3.91	1.998	1.57	1.283	2.30	1.828
n5OH_HEXANOIC	0.00	0.000	0.71	0.726	1.22	1.489
n2OH_ISOCAPROIC*	0.00	0.000	0.42	0.646	0.00	0.000
ACONITIC	28.52	11.528	16.42	7.919	12.28	7.522

*metabolites that were not consistently and statistically dys-regulated in cohort 2

Table 1. Urine metabolites that are differentially regulated in screening cohort 1. Using GC-tandem MS the urine metabolome was analyzed among 24 patients with type 2 diabetes and CKD and 11 of them were found to meet the statistical criteria of a total of 120 metabolites tested. The analysis was repeated in a larger cohort of patients with both type 1 and type 2 diabetes and CKD and found that of the 11, 8 of them remained statistically different. Of these metabolites, all were reduced in the diabetic cohorts except for n5OH-Hexanoic acid which was undetectable in the normal cohort and found to be increased with diabetes and CKD.

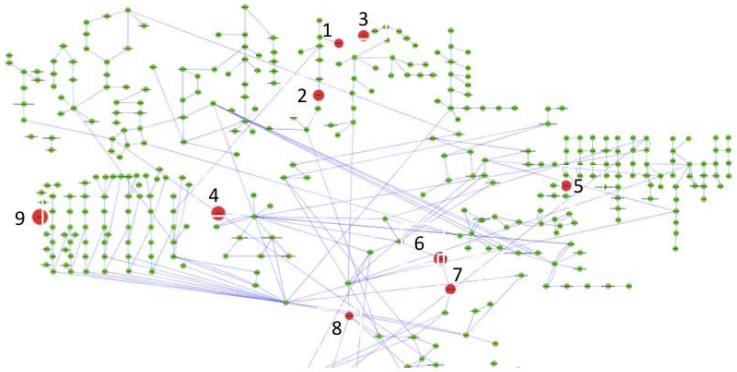

Figure 3. Compounds varying most significantly in human diabetic kidney disease. The metabolites which were found to have significant concentration differences between samples from control subjects (N=23) and two cohorts (N=14 and N=49) of diabetic patients are shown against a map of the basic metabolomic map of targeted species. Species with significant concentration differences are shown in red, displayed as circles with radius inversely proportional to the fold-change (i.e. all showing decreases, ranging from a factor 1/1.29 to 1/3.58). Species identified are (1) 2-hydroxyisobutyrate and (2) 3-hydroxyisobutyrate (intermediates in valine catabolism, (3) homovanillic acid, a catecholamine product, (4) glycolic acid, a compound arising from serine and pyruvate and participating in the metabolism of both the mitochondrion and peroxisome, (5) uracil, a pyrimidine base, (6, 7, 8) citric, aconitic and succinic acids, intermediates in the Krebs cycle, and (9) azelaic acid, an odd-chain, 7-carbon dicarboxylic acid arising from gut bacteria, reflecting the contribution of the microbiome.

These candidate metabolites should provide a perspective on the pathophysiology underlying diabetic nephropathy. The decrease in urinary citrate in patients with diabetic kidney disease may reflect either the described, whole-body increase in citrate utilization for cataplerosis and synthesis of intracellular lipid intermediates, or increased renal tubular reabsorption, or both. The increase in 5-hydroxyhexanoic acid, also known as 5-OH-caproic acid (5HCA), can be attributed to mitochondrial dysfunction, specifically defective mitochondrial fatty acid oxidation. 5HCA is a 6 carbon intermediate of medium chain fatty acid oxidation. The depletion of citric acid cycle intermediates proximal to oxoglutarate reflects a decrease in mitochondrial oxidative function, and an increase in mitochondrial cataplerosis. Uracil pools are regulated by *de novo* pyrimidine biosynthesis,the 4^{th} step of which is controlled by a mitochondrial enzyme, dihydroorotate dehydrogenase (DHOD). The activity of this enzyme is decreased whenever mitochondrial electron transfer to CoQ10 is decreased. All of these metabolomic clues suggest a consistent disturbance associated with diabetic kidney disease; carbon skeletons and electrons are shunted away from mitochondrial oxidation, resulting in increased cataplerotic production of amino acids (from oxoglutarate) and lipid (from acetyl-CoA, increased mass of peroxisomes and increased peroxisomal fatty acid, glycine and hydroxyproline oxidation, and depletion of the citric acid cycle. The panel of metabolites we have identified is novel and thus it would be important to determine their clinical significance.

Aim 1. Human urine metabolomics. Rationale and Experimental design The proposed studies in aim 1 will determine the functional significance of the altered metabolite profile in patients with diabetes and kidney disease. The panel of urine metabolites that are reduced in diabetic kidney disease is likely due to a shift in mitochondrial function from oxidation to cataplerosis. As reduced mitochondrial function will be the initial step to ultimate cellular dysfunction we hypothesize that the panel of urine metabolites will predict eventual decline in renal function as measured by the less sensitive measure of eGFR. We will evaluate two of the best characterized cohorts of patients with diabetes and longitudinal follow up of renal function: the FinnDiane cohort and the CRIC cohort. In both cohorts we will determine the role of each of the 8 metabolites to predict a decline in renal function and thus identify new biomarkers of importance to diabetic renal complications.

Aim 1a. Urine metabolomics and course of kidney disease in type 1 diabetics in the FinnDiane population. The levels of the candidate 8 metabolites that have been found to be differentially regulated in the diabetic urine will be assessed from urine collection in 1000 patients with type 1 diabetes at time of enrollment. The Finnish Diabetic Nephropathy (FinnDiane) study is an ongoing nationwide prospective

multicenter study in Finland established to identify risk factors for type 1 diabetes and its complications. The FinnDiane registry has been described in detail in previous publications [3]. 4,201 adults with type 1 diabetes were recruited from hospitals and primary health care centers across Finland. Fasting blood samples were obtained for the measurement of A1C, lipids, and creatinine, and estimated GFR was calculated by the CKD-EPI equation [4]. Baseline urinary albumin and creatinine were also measured. The presence of chronic kidney disease was defined by the presence of either microalbuminuria, macroalbuminuria, an estimated GFR <60 ml/min per 1.73 m2, or end-stage kidney disease. Hypertension was defined by antihypertensive medication use or a blood pressure greater than target levels of 130/85 mmHg. Since enrollment, participants have returned annually and provided repeat blood specimens for eGFR determination and urine specimens for albuminuria and biomarker analysis. To date, the average follow-up has been 6 years, with a range of 1-12 years. From the baseline measurements, we will determine what the prognostic value is of each of the metabolites to correlate and predict change in eGFR during the period of follow-up (2-6 years). We will evaluate 300 patients with longstanding type 1 diabetes and no evidence of albuminuria, 300 with microalbuminuria (ACR > 30mg/g) and 300 with macroalbuminuria (ACR > 300mg/g). 100 patients who went on to develop ESRD will also be assessed. Each group will be matched for age, sex, and duration of diabetes. Of the 4201 patients recruited into the study 2,296 did not develop albuminuria, 504 developed microalbuminuria, 580 developed macroalbuminuria and 293 developed end-stage kidney disease. From this large, well characterized cohort we will identify participants as described above and determine the relation of urine metabolites with longitudinal decline in eGFR over an average of 6 years.

Aim 1b. Urine metabolomics and course of kidney disease in type 2 diabetics in the CRIC study. The Chronic Renal Insufficiency Cohort (CRIC) study is a multi-center observational cohort which enrolled patients from 7 clinical centers (13 total sites) located throughout the US. Patients with reduced GFR (Stages II-IV CKD) between the ages of 21 and 74 were eligible for study participation, and by design the study sought to enroll 50% of participants with diabetes [5]. The study successfully enrolled 3939 participants, 48% of whom have diabetes (predominantly type 2). The age and eGFR criteria were specifically designed to facilitate evaluation of the progression and implications of CKD across a wide spectrum of mild to moderate kidney dysfunction and age. The level of eGFR used to define eligibility was based on the four-variable Modification of Diet in Renal Disease (MDRD) estimating equation, using locally measured serum creatinine calibrated to the Cleveland Clinic laboratory. CRIC participants are followed until death or withdrawal of informed consent. Hypertension at entry was defined as either systolic BP ≥140 mmHg, diastolic BP ≥90 mmHg, or use of antihypertensive medications. Diabetes was defined as either fasting glucose ≥126 mg/dl, random glucose ≥200 mg/dl, or use of insulin or antidiabetic medication. For this study, we will recruit 1000 CRIC participants with diabetes who will be equally divided by CKD stages 3a (eGFR 45-60), 3b (eGFR 30-45) and 4 (20-30). We will measure the urine metabolites in the first 24h urine sample and determine whether the urine metabolites are associated with longitudinal decline in eGFR. Patients have yearly GFR measured based on serum creatinine and are followed up for a median of 5 years. Additionally, urine samples are available at yearly intervals at year 1, year 2 and year 3. Changes in the level of metabolites will be analyzed in relation to eGFR change in subsequent years.

Analytic Plan The analytic plans are very similar for both the FINN-DIANE and CRIC study. We plan to evaluate associations separately within each cohort, given differences in type 1 and type 2 diabetes, and do not plan to meta-analyze the data across the two studies. Standard descriptive statistics will be used to characterize the overall study population and subgroups of interest both at baseline and during follow-up. Summary statistics such as means, medians, standard deviations, and ranges will be produced for measured variables. Frequencies will be tabulated for categorical and ordinal variables. Graphical methods will be used to examine distributions, identify potential influential points, and guide in data transformations if warranted. We expect that a natural logarithm transformation will be applied to urine metabolite levels because of skewed distribution. For outcomes collected longitudinally, and to examine associations among various measures, scatter plots and grouped box plots will be produced to examine assumptions of linearity, symmetry, and homoscedascity. Next, we will estimate GFR (eGFR) using the CKD-EPI collaboration equation [4] at each time-point. We will use slope-based analyses to analyze the progression of renal disease. Compared to failure time analysis using clinical endpoint, e.g., ESRD, slope-based analysis uses all available information on GFR and so is likely to provide more power to detect differences between groups. Thus, it is likely to be of particular importance in studying subtle changes in renal function, and in studying the subgroup of subjects who begin

with higher levels of GFR. We will consider stratified analyses by baseline GFR level and check whether the associations between urine metabolite levels and change of GFR are modified by participant's GFR level. Initial models will be adjusted for age, sex, and race/ethnicity. A subsequent model will additionally adjust for variables that are known determinants of loss of kidney function in diabetic nephropathy (baseline eGFR, albumin/creatinine ratio, systolic blood pressure, use of ACE or ARB medications), to determine whether the urine metabolite profile remains associated with longitudinal declines in eGFR independent of these factors.

Power Calculation Sample sizes, SD of eGFR, and correlation between two repeated eGFR measurement one year apart are similar for both cohorts. Thus, evaluation of statistical power is also similar, and is presented only once here. We are interested in detecting the change of slope GFR per year when the predictor changes, e.g., one standard deviation increase of urine metabolite level. The calculation of detectable differences in slope depends on several factors; the standard deviation (SD) of GFR at one time point, the correlation of repeated measures within subjects, sample size, variance of the exposure, the number and timing of GFR measures, the alpha error, and power. We plan to measure urine metabolite levels using baseline samples in FinnDiane. At present, the mean follow-up in FinnDiane is 6 years (range 1-12 years) and 5 years in CRIC, with an anticipated average follow-up of at least 6 years by the time of this study. We will use an average of four and six GFR measures in the power calculation. Preliminary data from CRIC provides an estimate of SD of eGFR of 14 ml/min/1.73m^2 and the correlation between two repeated GFR measurements one year apart to be 0.80. On the basis of our preliminary data, we have an a priori hypotheses that 8 urine metabolites will be associated with longitudinal decline in eGFR, however, at no additional expense we plan to measure all available urine metabolites. Testing this number of predictors will increase the chance of a type 1 error. Therefore, we show power for both extremes – evaluating the standard two-sided alpha of 0.05, and also after employing a Bonferroni correction for 100 predictors (0.05/100 = 0.0005), keeping the overall type I error rate below 0.05. Table 2 displays the detectable slope differences at a sample size of 1000 assuming a 6% annual loss to follow-up rate with either 4 or 6 eGFR measurements and both two-sided type I error rates and power of 0.80. These data indicate that even with the most conservative estimate of only 4 available eGFR measurements and a type 1 error rate of 0.0005, we will have power to detect a slow of eGFR change of 0.34 ml/min/1.73m^2 per year or greater.

Table 2. Detectable eGFR slope differences per one standard deviation increase of exposure variable*

# of Annual eGFR Measurements	Type 1 Error Rate	Minimum Detectable eGFR slope (ml/min/1.73m^2 per year)
4	0.05	0.19
4	0.0005	0.34
6	0.05	0.11
6	0.0005	0.19

* Power = 0.80, Assumes initial sample size of 1000 individuals, and 6% annual loss to follow-up rate.

Urine Metabolomic Methods: Urine samples will be processed for metabolite profiling by GC-MS and LC-tandem MS. Only 1 ml of urine is required for these studies. For GC-MS, we will follow the silicic acid batch elution method developed in San Diego for urine organic acid analysis [6], as we have carried out for the preliminary data. In addition, we plan two LC-tandem MS runs for each sample: one in positive and one in negative ionization. To allow for detection of changes in novel or untargeted metabolites, we plan to use a hybrid quadrupole-time of flight (Q-TOF) instrument. Our list of targeted compounds begins with the 174 compounds that we presently quantify in separate platforms, including organic acids, amino acids, acycarnitines, and purines and primidines. We have also added several other compounds to our standard m/zes and plan to successively extend the list of targets. In addition, the Q-TOF will permit us to carry out untargeted metabolomics, The LC-MS profiling data will be analyzed for distinguishing features with XCMS software. This program performs nonlinear alignment and normalization of multiple data sets, and outputs a list of significant differences (m/z values, corrected retention times and integrated intensities), along with p-values from their Student's t test results. The most significant ions will be calculated by their p-values and their absolute intensities (to provde a S/N of >10), and those ions will be targeted for future identification. For these ions, accurate mass information will be used to calculate probable elemental composition, and the metabolites

identified with the following databases, NIST, KEGG, HumanCyc, ARM and METLIN. For the group of interesting metabolites, this information, along with collision induced dissociation (CID) data, will be further used for identification.

NIDDK deliverables and pitfalls: In these set of studies, we will establish the significant and clinically relevant role of a new set of urine biomarkers that may predict progression of renal disease in patients with both type 1 and type 2 diabetes. We will utilize possibly the best characterized cohorts in the world and utilize the most quantitative assessment of these urine metabolic markers. Coupling the technical expertise with outstanding mitochondrial and clinical expertise will lead to robust connections between the urine metabolome and kidney function. The results would thus open up new avenues for diagnostic and therapeutic approaches to gauging susceptibility in subjects at risk for DN. In future studies, we will explore newer aspects of the metabolome (e.g. lipidomics) for new candidates as our methodologies continue to evolve. In our statistical analysis we have considered methods to handle informative missing GFR due to drop-out. Ignoring the mechanism of missing GFR will create bias because shorter follow-up is typically associated with deeper decline in GFR. When a variable like GFR is known to be below some threshold, methods for censored data may be used. One can use a censored regression model (known in survival analysis as the accelerated failure time model) to model the association of time and other predictor variables with GFR in an appropriate fashion. The variance of the parameters will be estimated using robust variance estimation/GEE to take into account the multiple observations of GFR within subjects. When no information on GFR is available, we will consider shared parameter modeling approach. In this approach, we will explicitly model drop-out mechanisms in addition to the model for the repeated GFR measures. Repeated GFR measures can be modeled using linear mixed effects model. The dependency of drop-out on GFR decline will be introduced by allowing some common parameters in the two models.

Aim 2. Human urine epigenetics. A newly emerging area of discovery in genetic determination of complex traits is the realm of epigenetics, or heritable changes in the DNA microenvironment leading to changes in gene expression, which may be altered in susceptible cells in the setting of environmental stressors, such as hyperglycemia. For example, differential methylation at proximal promoter 5'-CpG-3' sites may selectively diminish gene expression *in cis*. Here we will exploit DNA in urine sediment, as a source of DNA reflecting events in renal tubular epithelial/podocytes from the kidney. We will initially focus on PGC1α methylation of its promoter as PGC1α is a master regulator of mitochondrial biogenesis. Gene expression and protein levels of PGC1α were reduced in our studies with the diabetic kidney (Fig. 4 A, B). Barres et al [7] have shown cross-sectional differences in CpG methylation in skeletal muscle from diabetic cases, versus controls; their findings suggested selective methylation of the PGC1α locus, thereby diminishing PGC1α expression, resulting in diminished mitochondrial abundance.

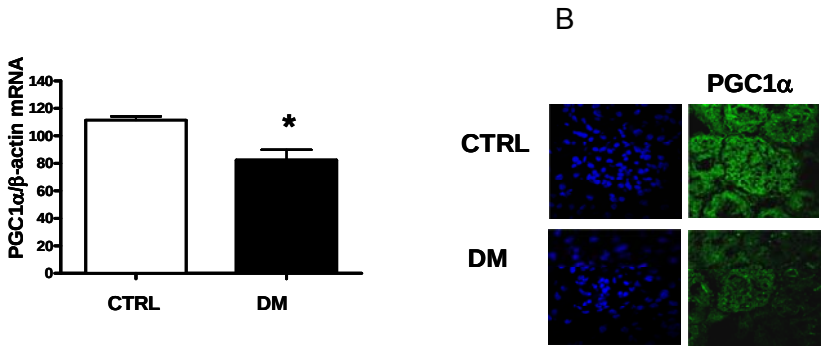

Figure 4. PGC1α in the diabetic kidney. (A), PGC1α is reduced in diabetic kidneys as demonstrated by real time PCR analysis of control and diabetic kidneys (n=6 per group, *p< 0.05) and **(B)** immunostaining with an antibody to PGC1α.

For **Aim 2**, our Co-PI, Daniel O'Connor and collaborator Bing Ren have developed methods to assess the epigenetic alterations of promoter methylation in the blood and urine of humans. We have successfully prepared genomic DNA from both blood and urine (tubular epithelial and podocytes) for bisulfite sequencing at CpG sites in candidate genetic loci, as well as the global (genome-wide control) LINE-1 promoter, in 48 subjects. Extensive CpG methylation in a promoter is generally associated with inhibition of transcription of that gene. In one study of susceptibility to/outcome in AKI (Acute Kidney Injury), we found that kallikrein-1 (KLK1) promoter CpG methylation was reduced (compatible with increased gene expression) in urine DNA, while

LINE-1 promoter methylation remained elevated, as a control. In patients with AKI, there was a rise in KLK1 promoter CpG methylation (case versus control; see Figure 5), thereby tending to diminish KLK1 expression. These studies demonstrate the feasibility of performing urine DNA methylation studies in patients.

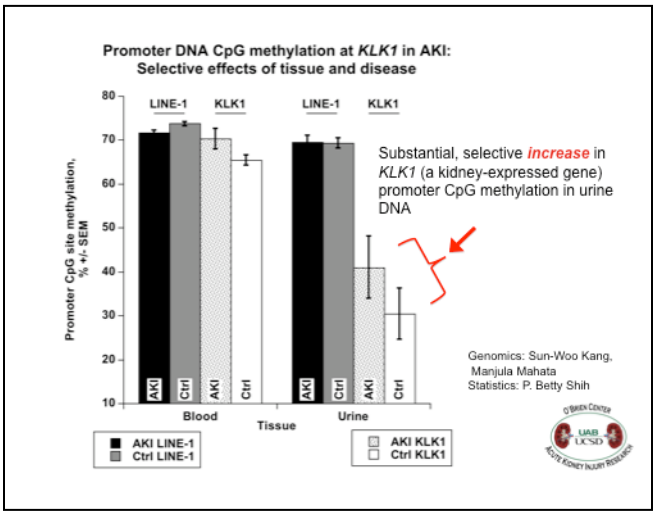

Figure 5. CpG methylation analyzed by bisulfite sequencing: Results at the *KLK1* promoter, as well as a global control (LINE-1 repetitive elements), in genomic DNA from urine or blood. Results (shown as mean ± one SEM), from established AKI cases or healthy controls, were analyzed by ANOVA, factoring for age, sex and race. Promoter *KLK1* specific methylation was substantially higher in blood than urine DNA (blood 66.38±1.00 vs. urine 33.43±4.67%; ANOVA p<0.0001). Promoter *KLK1* methylation in blood DNA was higher in AKI than controls (AKI, 70.32±2.27 vs. controls, 65.36±1.05%; ANOVA p=0.011). Promoter *KLK1* methylation in urine DNA did not differ in AKI versus controls (AKI, 40.95±7.06 vs. controls, 30.35±5.88%; ANOVA p=0.22).

Experimental design: Timed urine samples frozen at –70°C before assay will be obtained. Urine DNA is prepared from the urine pellet with spun columns (urine DNA isolation kit; Norgen, Canada). Urine DNA is subjected to sodium metabisulfite ($Na_2S_2O_5$) treatment (Imprint™ DNA Modification Kit; Sigma, USA), and then eluted in 20 µL elution buffer. Bisulfite converts cytosine residues to uracil, but leaves 5-methylcytosine residues unaffected. In urine or blood DNA at candidate pathway loci (including PGC1α), we can employ selective restriction digestion at the 5'-CCGG-3' recognition site by the isoschizomers HpaII (which cannot cut when C is 5-methylated) versus MspI (which ignores C-methylation), followed by qPCR, to quantify % methylation of promoter CpG sites. This procedure allows reliable quantitation of CpG methylation even in sparse urine DNA. The urine sediment obtained in this way is also amenable to rapid chromatin isolation followed by ChIP (Chromatin ImmunoPrecipitation) analysis, probing promoter interactions with endogenous factors such as candidate transcription factors, or interrogation of histone modifications (acetylation, methylation) as another approach to epigenetic control of gene expression.

***P*romoter CpG methylation analysis.** Pyrosequencing for allele discrimination (Pyrosequencing; Qiagen, USA) provides real-time extension-based DNA analysis that can evaluate multiple CpG sites [8]. CpG methylation analysis at the 5'/upstream/proximal promoter region of the primary target gene (*PGC1A*) is performed. The gene is analyzed by a single PCR amplicon spanning multiplle CpGs in a 150-250 bp region, with two biotinylated sequencing (extension) primers. As a control, global CpG methylation analysis is completed using PyroMark LINE-1 reagents (Pyrosequencing; Qiagen, USA). We thus determine the methylation status of three CpG sites in LINE-1 repetitive (LTR-like) elements, wherein methylation levels of CpG sites represent global methylation status across the genome, because of the repetitive nature of LINE-1 elements [9]. The PCR will be performed with one of the PCR primers biotinylated to convert the PCR product to single-stranded DNA template. PCR products (each 10 µl) will be sequenced by Pyrosequencing PSQ96 HS System (Pyrosequencing, Qiagen, USA). The methylation status of each locus will be analyzed individually as a T/C SNP using Pyro-QCpG™ software (Pyrosequencing).

Epigenetic data analyses. CpG site methylation is a continuous trait, typically scaled from 0→100%. Results are expressed as the mean value ± one standard error of the mean (SEM) for continuous variables. For comparisons of two groups, unpaired two-sided *t*-tests or one-way ANOVA (enabling adjustment for covariates of age, sex, and ethnicity) is performed. Non-parametric Wilcoxon Rank Sum test is used to confirm parametric tests in the face of relatively small sample sizes. Proportions are evaluated by Fisher's exact test (2x2 tables) or chi-square test (3x2 tables). Statistical analyses are performed in R2.10.1 <http://www.r-project.org/> or SPSS-17 (Statistical Package for the Social Sciences; Chicago, IL, USA). A *P* value of <0.05 is considered significant. Multiple linear regression is performed with default criteria of entry (p<0.05) and exit (p>0.10) from the multivariate regression model, using stepwise or forward options. Statistical power for genetic studies is approached by the methods of Shaun Purcell and Pak Sham <http://pngu.mgh.harvard.edu/~purcell/gpc/>).

Realistic power calculations are necessarily difficult and imprecise, since the necessary assumptions in such calculations may not be known *a priori*. Nonetheless, a spectrum of power calculations can be done, varying the assumptions over realistic ranges of possibilities. Our primary aim will be to study enough individuals (DN case versus non-DN control) to achieve significant association, assuming alpha<0.00002, power=0.8, V_A ~0.3 (i.e., heritability of ~30%). We likely will have reasonable power for trait associations to CpG methylated loci, if the locus-specific heritability is relatively high and the marker CpG is present in the promoter, and functional.

NIDDK deliverables and pitfalls: Specific regions of PGC1α promoter methylation will be assessed in a well characterized cohort of patients with type 1 diabetes and progressive CKD. The relationship of promoter methylation with other functional assays (urine ACR and other clinical outcomes will also be evaluated). Coupling promoter methylation studies with the metabolomic profile will be of enormous interest as well. In the present study we will not perform genome-wide patterns of methylation in the subjects as there will be many leads that may not be relevant to renal pathology. A whole epigenome approach will be pursued in aim 3 in the mouse model and based on our systems biology analysis, additional methylation targets will be evaluated in the human urine. The results would thus open up new avenues for diagnostic and therapeutic approaches to gauging susceptibility in subjects at risk for DN. Promoter CpG methylation analysis requires DNA of sufficient quality and quantity for either sequencing post-bisulfite, or restriction digestion. We have therefore performed pilot experiments, and found that post-bisulfite pyrosequencing works well at candidate loci in urine DNA. As a backup strategy, we have also implemented the less harsh (i.e., no bidulfite) technique of differential restriction digestion at CpG sites (by the isoschizomers HpaII versus MspI) followed by qPCR with SYBR-green primers.

Aim 3. Experimental animals: Superoxide imaging, mitochondrial function, metabolomics and epigenomics.

Overturning the Paradigm of Excess Mitochondrial Superoxide production in Diabetes. As noted in live animal studies (Figure 1) we found that diabetic mice exhibited reduced renal superoxide anion production. To confirm our results and evaluate specific tissue compartments, studies were performed by administration of DHE 16h prior to euthanasia as previously described [10,11]. Confocal imaging of slices prepared from animals injected with DHE also demonstrated that control mice had significantly higher superoxide production in kidney cortex and glomeruli than diabetic animals (Fig. 5 A, B, C). These data further demonstrate that the diabetic kidney *in vivo* exhibits lower, not higher superoxide production, than normal kidneys.

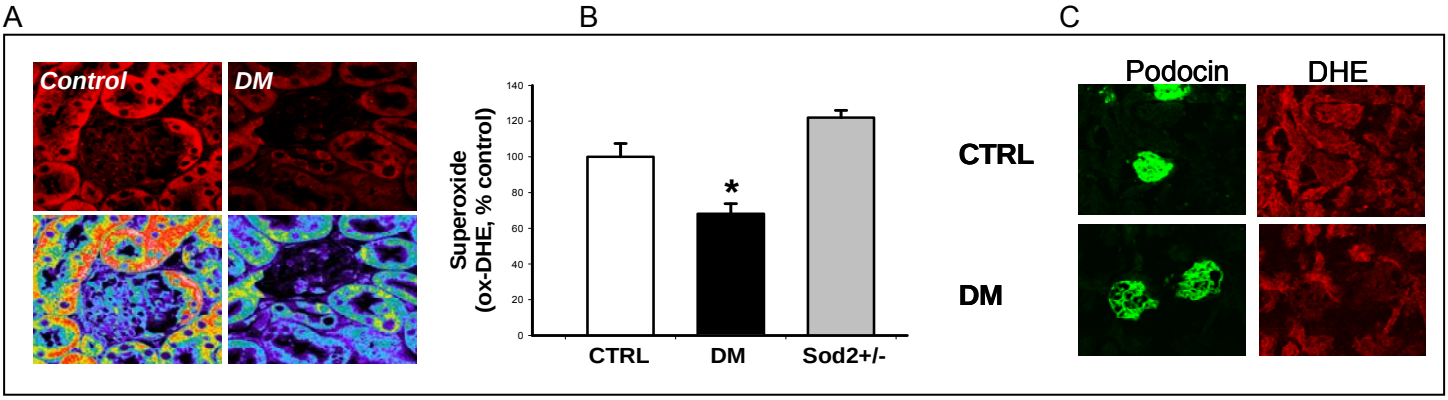

Figure 5. Reduced superoxide production in diabetic kidneys (**A),** ox-DHE fluorescence (red, top; linear pseudocolor,bottom) in kidney slices prepared from DHE-injected control and DM mice with less *in vivo* oxidation in DM kidney. (**B),** Quantitative analysis was performed using MetaMorph6 image analysis software by an analyst blind to treatment group. To demonstrate that increased mitochondrial superoxide could indeed be measured if present; kidneys from *Sod2*$^{+/-}$ mice which are deficient in mitochondrial superoxide dismutase were evaluated and demonstrated the expected higher superoxide than controls. (n ≥ 15 each for control and diabetic groups, n=3 for SOD 2+/- group, *p<0.05 vs control). (**C),** Slice imaging of perfused kidneys from DHE-injected control mice exhibited DHE oxidation in glomeruli, as identified by immunostaining with the podocyte specific antibody, podocin. Diabetic kidneys had a reduced level of glomerular DHE oxidation-derived fluorescence**.**

To confirm that increased mitochondrial superoxide production, if present, could in fact be visualized by our *in vivo* DHE method, *Sod2+/-* mice exhibited the expected increase in DHE oxidation (Figure 5B). Mitochondrial production of superoxide anion *ex vivo* from kidney cortical homogenates from diabetic animals via electron paramagnetic resonance (EPR) did not demonstrate an increase in superoxide production (Fig. 6). In addition, direct addition of glucose (25 mM) did not increase superoxide levels in the control or diabetic animals (Fig. 6). The EPR signal for superoxide was fully blocked by SOD further confirming the identity of the ROS being detected (data not shown).

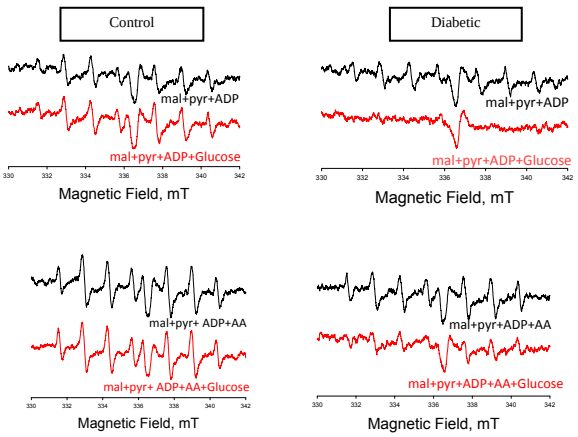

Figure 6. EPR spectra in kidney homogenates from control and diabetic mice. Diabetic kidney cortex (right panels) did not show evidence of increased superoxide anion production when evaluated ex vivo by EPR. Addition of the mitochondrial substrates malate and pyruvate in the absence (upper panels) or presence (lower panels) of antimycin A (AA). Addition of high glucose (bottom traces) did not increase mitochondrial superoxide production in samples from control animals (left panels) (n=6) with or without antimycin A, and reduced the level of superoxide in the diabetic samples (right panels) (n=6) with or without antimycin A, a positive control for the sensitivity of the assay.

Our theory does not discount other sources of ROS such as NADPH oxidase, excess nitric oxide, and xanthine oxidase pathways. However, these other sources of ROS are potentially downstream of a persistent Crabtree and Warburg effect and not due to increased mitochondrial superoxide anion production. Our preliminary experiments are the first studies that we are aware of that have used DHE *in vivo* in an animal model of diabetes. We have also adapted EPR to follow free radical dynamics in tissue homogenates and cells from several organs [11,12]. By comparing DHE and EPR data in cells and tissue samples from the same cohort of animals used for fluorescence imaging our multi-disciplinary team will obtain an integrated picture of the specific ROS species that are produced, the time course of production and the cell-types in which ROS are generated in response to type 1 diabetes. Additional studies will examine mitochondrial complex activity and mitochondrial oxygen consumption to give a comprehensive analysis of mitochondrial function in the development of diabetic kidney disease in two contrasting models. Studies with *in vivo* metabolomics and epigenetics will identify additional methods to measure mitochondrial function from kidney and urine.

Mouse models of type 1 diabetic kidney disease: It is acknowledged that there are presently no ideal models of human diabetic nephropathy, however several models exist that recapitulate several features of early diabetic kidney disease. The two models that will be examined in the current proposal are the Bl6J Akita mouse as a model of early but not progressive diabetic kidney disease and the Akita F1 cross between DBA and C57Bl6J as a potential model of progressive diabetic kidney disease [13,]. The advantage of studying both models of type 1 diabetes is that we will be able to contrast the metabolomic profile in two models and determine if albuminuria (a critical clinical feature of human diabetic nephropathy) is associated with differences in the urine metabolome profile. In addition, we will study the PGC1α floxed mouse. As our overall hypothesis is that reduced mitochondrial biogenesis is the major basis for the initiation of pro-inflammatory and pro-fibrotic changes in the diabetic kidney, we expect to demonstrate that reduction of PGC1α specifically in podocytes will result in a rapid glomerular disease in the Akita Bl6 mouse background. We have both the PGC1α floxed mouse and the podocin-Cre mouse in the Bl6 background and have already generated litters. Evaluating these various mouse models (Akita Bl6, Akita F1 cross, and the Akita podocyte deleted PGC1α) with the appropriate controls will provide strong evidence that reduction of mitochondrial function and biogenesis plays a critical role in progressive diabetic kidney disease. The mouse models of diabetes will be studied along with non-diabetic controls from littermates in the same background at 6 weeks of age and 6 months of age. There will be 30 mice per group. 24h urine collections will be collected at each of the time points in the week prior to euthanasia. All mice will be phenotyped at the histologic and gene expression and protein levels. 10 mice in each group will be targeted for image analysis. 20 mice from each group will undergo analysis for mitochondrial function, metabolomics and epigenetic studies.

In vivo imaging of superoxide levels and superoxide detection by EPR To measure superoxide production *in vivo*, we will administer the superoxide-sensitive fluorescent compound, DHE, as described in detail [11,14]. Briefly, mice receive two intraperitoneal (i.p.) injections, spaced 30 minutes apart, of 200 μl of injectate (27 mg/kg DHE per injection). Two imaging techniques are then used to evaluate DHE oxidation *in vivo*. The first will be whole-animal imaging in a GE eXplore Optix 100 scanner in the UCSD Moore's Cancer Center. The second technique will image and analyze DHE oxidation in fixed slices prepared from animals injected with DHE, using confocal imaging as we have previously described [11,14] (and as above). The average DHE-oxidation fluorescence intensity for each cell of interest is logged, and values averaged to determine the mean DHE oxidation-derived fluorescence. To measure ROS and superoxide anions directly, the reaction mixture (tissue homogenate, isolated mitochondria, isolated glomeruli) will be injected into the EPR cavity through a gas-permeable Teflon tube, which allows control of [O_2] in the samples. The sample mixture also contains 10-100 mM DIPPMPO or DEPMPO (5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide), and the desired concentration of specific substrates and/or inhibitors, and the mixture is then pushed through the Teflon tube into the EPR cavity, where the EPR spectrum is recorded. These studies will be carried out in a MiniScope MS200 Benchtop EPR Spectrometer (Magnettech, Germany) which is designed to allow tight control of pO_2 and temperature. Calibration of EPR signal intensity to [paramagnetic species], e.g. $O_2^{\bullet-}$, are carried out using the stable radical, 4-hydroxy-TMPO.

Tissue and urine metabolomics coupled to mitochondrial function and oxygenation We will determine if the urine metabolites correlate with renal changes of the same metabolites, and mitochondrial changes as assessed by functional and structural methods. Whole tissue metabolomics will be performed in specimens snap frozen in liquid nitrogen for organic acid analysis by GC/MS, tissue (intracellular) lipid quantitation as neutral fat and cholesteryl-esters, and tissue glutathione and lipid peroxide quantitation and mitochondrial respiratory chain function (electron transport complex assays, citrate synthase), catalase assay as a measure of peroxisomal mass, mtDNA copy number and damage, as previously published [15-18]. Further indications of mitochondrial function can be gained through measurement of rates of oxygen consumption by mitochondria isolated from the kidney cortex and by intact glomeruli. The isolated mitochondrial experiments can provide insight into whether particular oxidative pathways are dysfunctional because the oxidizable substrates offered to the mitochondria can be controlled and compared (i.e. pyruvate/malate, glutamate/malate, succinate with rotenone, and acetoacetate/beta hydroxybutyrate). Assessment of respiration in intact cells is more physiological and reflects a net balance between energy production and energy demand, but fewer variables can be controlled, such as overall energy demand and substrate/ADP provision to the mitochondria. The combination of these approaches can give powerful insight into bioenergetic mechanisms that underlie tissue dysfunction. Kidney mitochondria will be isolated as previously described [2]. Intact cell respiration of renal kidney cortical tubules and glomeruli will be performed essentially as described by Beeson et al. [19] using 40,000 cells/well in the XF24. Dr. Murphy works closely with Seahorse scientists and has developed methodology to use isolated mitochondria in the Seahorse (an apparatus designed for use with monolayers of intact cells) (http://www.seahorsebio.com/webinars/webinar-2009-10-28-murphy.php).

Global CpG methylation profiling and mechanistic studies of promoter methylation of PGC1α. For more systematic global (genome-wide) changes in CpG methylation in genomic DNA obtained from mouse kidneys, we will employ the Illumina Infinium HumanMethylation-450 BeadChip Hybridization system, in which ~450K promoter CpG sites are probed simultaneously, providing coverage of RefSeq genes, including promoter, 5', and 3' regions, using 500 ng of bisulfite treated genomic DNA per sample. The assay interrogates these bases using two site-specific oligonucleotide probes, one for the methylated locus and the second for the unmethylated locus. To determine functionally important polymorphisms in the promoter (non-coding) regions of candidate genes in renal cell types, we will use the methods we have reported recently for the effect of human CHGA promoter polymorphisms on CHGA gene expression in PC12 cells by us [20]. As a unique advance for these promoter CpG studies, we will explore the mechanistic significance of CpG methylation in transcription, inserting candidate promoters into a newly engineered series of CpG promoter/luciferase reporter plasmids (pCpGL-Basic), transfected into appropriate cell types. The pCpGL-Basic vector has all CpG sites removed from the backbone, leaving the only (potential). Prior to transfection, the plasmid is treated with either DNA methyltransferase, demethylase, or mock/buffer. In this way, the effect of CpG methylatiion on transcription can be quantified.

Systems Biology for analyzing large dataset for diabetic complications. Experiments done in metabolome analyses (Aim 1) and epigenetic analyses (Aim 2) will produce huge amount of data which are complicated and thus it is very difficult to make biological interpretation for these data manually. In systems biology project of this research, we make the most of computational resources to handle these data and to assist such biological interpretations; we will efficiently integrate various types of data to place each datum onto biological context such as metabolic pathways and transcription regulatory networks. These data include not only those obtained from our experiments but may also include publicly available data. Systems biology project will especially focus on biological phenomena at molecular level and attempt to make connections to disease phetotypes, i.e. stages of diabetic kidney disease. To do so, our systems biology experts (Ideker, Saito) will combine the metabolome analyses and epigenetic analyses to build new models of specific biological mechanism [21,22]. In collaboration with metabolome analyses, systems biology project will conduct biological network analyses. After measuring levels of each metabolite in urine from FinnDiane cohort and CRIC cohort by GC/MS, each of them will be mapped onto known biological pathways to infer associated pathways for the specific phenomenon. If we could find changes in metabolites which are located closely to each other on the pathway, it would be the strong evidence that the pathway is associated with the phenomenon. Integration of various biological networks such as protein-protein interactions or protein-DNA interactions may be efficient in some cases to find novel signaling pathways which connect metabolites showing coordinated changes. Metabolic pathways may be handled by Cytoscape by representing metabolites or enzymes as nodes and their connections by edges, respectively. Changes in the concentration of each metabolite can be represented as color of the node. In addition, the plug-ins for Cytoscape allows one to perform specific tasks such as collection of biological networks from the literatures or from public databases. The plug-ins have been developed by large community of Cytoscape developers and additional plug-ins to perform necessary tasks. Changes in the metabolome can be efficiently characterized by mapping these changes to the biological network as we have shown in our previous works that breast cancer metastasis can be predicted with high reproducibility by integrating gene expression data onto protein-protein interaction networks. In collaboration with epigenetic analyses, systems biology project will conduct biological sequence analyses to elucidate possible transcription regulatory machinery behind diabetic diseases, especially focusing on regulatory machinery around PGC1α. After obtaining global methylation patterns among mice with various stages of diabetic kidney disease, we will analyze not only methylated genomic regions but also their sequence patterns in methylated regions, putative promoters associated with methylated regions, and expression levels of the associated genes will be analyzed to infer possible transcription networks governing phenotypes of the cells and/or tissues. This could be done efficiently as we have worked on epigenetic analyses of nucleosomes in various regions of the genome to find associated sequence patterns [23]. The new methylation sites from the mouse studies will then be queried with the human equivalent in our urine samples from the type 1 diabetic population.

NIDDK deliverables and pitfalls: The overall results from aim 3 and in conjunction with aims 1 and 2 will identify the role of mitochondrial biogenesis in diabetic kidney disease. The mouse studies will establish the relationship between urine metabolomics and epigenetics with kidney superoxide anion production, mitochondrial complex activity, mitochondrial oxygen consumption and markers of diabetic kidney disease in non-progressive and progressive models of diabetic kidney disease. Specific studies with the methylation of the PGC1a promoter will demonstrate the functional significance of the methylation in cell culture systems. However, successful promoter/reporter studies will not establish the mechanism whereby a variant alters gene expression; the next stage will include EMSA with variant oligonucleotides (including CpG monomucleotides) and appropriate cell type nuclear extracts; if a candidate factor is likely upon motif analysis (TransFac searchable at “Promolign” web interface <http://polly.wustl.edu/promolign/main.html>), we perform appropriate antibody supershifts. Finally, to verify that the trans-factor/cis-promoter interaction occurs in vivo (in cells), we will undertake ChIP analyses, also in the appropriate cell type. The whole epigenome will be evaluated in the tissue and urine from the mouse studies. Data will be analyzed as in Aim-2 (human epigenetics), with the caveat that multiple testing must be accounted for in such global genomic analyses. Instead of a typical genome-wide threshold for multiple testing (e.g., threshold $p<5x10^{-8}$), we will rely on replication as the ultimate guardian against false positive conclusions. A systems biology approach will determine the relevance of the relationships between epigenomics, metabolomics and specific functional and pathologic phenotypes. By having samples from large well characterized cohorts, the clinical relevance of new findings will be translated back into humans.